**Characterization of feline Borna disease virus**

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For the two most important people in my life, my loving parents, Mr & Mrs Oladele.

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Feline Borna disease, a neurological disease of cats is an infection caused by Borna disease virus (BDV). The infection, first reported in Sweden in 1974, affects other species of animals such as horses in which the disease was first reported. Sheep is the other natural host of the virus, but BDV infection is also seen in cattle, dogs, goats, donkey, and some zoo animals. The disease is widely speculated to be zoonotic as it is believed to affect humans as well. In Sweden, the infection is endemic in central part of Sweden namely in the Stockholm and Uppsala area, where numerous cases have been reported in the past and new cases continue to emerge in recent times. The importance and continued incidence and prevalence of the infection in cat prompted this present study. Archive and incoming samples from cats, horses and dogs were screened by real- time RT-PCR. Eight positive cat samples were detected by real-time RT-PCR. Three of these samples were used for virus isolation and characterization, while two other positive samples were used to test previously described primers. In conclusion, we have been able to detect eight positive samples from the cat by real-time RT-PCR. Though the virus was not detected upon sub-passages in vero cells, demonstration of presence of the virus from passage 10-12 in the positive control show that other factors than the assay may have contributed to the lack of virus isolation The classical PCR for molecular epidemiology needs further optimization as reflected in the fact that only three primer pairs seem to work with the C6BDV control
**Keywords**: Borna disease virus, rRT-PCR, feline Borna disease, Molecular epidemiology

# Contents

## Background

Viral infections such as rabies, cowpox and smallpox have been known to affect domestic animals and humans for ages. These infections have deleterious effects on the health and wellbeing of humans and animals and are of grave economic importance. They pose serious public health significance not only to individuals and animals but also to the society in general. Borna disease (BD), a fatal T-cell mediated immunopathological disease affecting the central nervous system, was originally recognised in horses and sheep in certain areas of Germany (Durrwald & Ludwig, 1997), where the disease caused large scale epidemic resulting in the death of several thousands of horses. BD has also been diagnosed in other species of animals such as rabbit, cattle, dog and certain zoo animals (Bode *et al*., 1994; Lundgren *et al*., 1995) and a paraetic syndrome in ostrich has been reported in Israel (Malkinson *et al*., 1995).

Cases of nonsuppurative meningoencephalitis in domestic cats have been reported in Austria, Sweden, Japan and several parts of the world (Helps *et al*., 2001; Lundgren *et al*., 1995; Nakamura *et al*., 1996; Nowotny & Weissenbock, 1995; Reeves *et al*., 1998). The present project focussed primarily on the detection and characterization of feline Borna disease virus in Sweden. The emphasis in this study was the use of molecular techniques to gain a better understanding of BDV, in order to address the situation as regards feline Borna disease in Sweden.

## Introduction

### History

In the 18th and 19th centuries in southern Germany, clinical signs in horses similar to that of Borna disease were first described. This was the first record of its kind as regards to BD in any part of the world. There had been an epidemic like outbreak in the 1890 and the first decade of the 20th century in the city of Borna, near Leipzig, Germany, where a large number of horses had died from the disease. The name Bornasche Krankheit (Borna disease) was coined from the district around the city where the epidemic had occurred. In the 1920s, the aetiology of the disease was demonstrated to be a virus (reviewed in Durrwald & Ludwig, 1997; Ludwig & Bode, 2000). As early as the 1950s, feline cases of nonsuppurative meningoencephalitis had been reported in different parts of the world (Hoff & Vandevelde, 1981; Mcgaughey, 1953; Truyen *et al*., 1990). At that time the disease was poorly defined and the aetiology although unknown, was assumed to be of viral origin. However, in 1974, a feline neurological disorder of cat with an unknown aetiology was described in Sweden (Kronevi *et al*., 1974). Cases of feline meningoencephalitis commonly called vingelsjuka in Swedish (staggering disease) continue to occur in mid-Sweden even till date. In the 1990s, staggering disease was linked to BDV infection (Lundgren *et al*., 1995).

## Etiology

The etiological agent, Borna disease virus (BDV), is a single-stranded negative-sense enveloped non-segmented RNA virus (Briese *et al*., 1994; Cubitt *et al*., 1994) and is the prototypic member of the new family *Bornaviridae,* within the order *Mononegavirales* (De la Torre, 1994; Schneemann *et al*., 1995). The virus is believed to be an old evolutionary pathogen that has had time to adapt to a wide range of mammalian hosts. The virus is highly neurotropic, having affinity for the limbic system and the reticuloendothelial system.

## Unique features of BDV

BDV exhibits several unique features or characteristics, among them the genome organisation and nucleotide sequence that are typically similar to other members of the order *Mononegavirales*. Unlike other RNA viruses which replicate and transcribe in the cytoplasm, nuclear localization of replication and transcription is typical of BDV (Pyper *et al*., 1998). The unusually high level of sequence conservation (Formella *et al*., 2000) is unexpected for an RNA virus that has a high rate of replication and transcription and thus likely to mutate often.

The virus seems to have wider host range than previously thought and possibly affecting humans as well (Rott *et al*., 1985; Bode *et al*., 1995). The virus causes a persistent infection of the CNS with astrocytes primarily affected (Carbone *et al*., 1989 and 1993). A characteristic feature of the infection is the development of a prominent astrocytosis (Gonzalez-Dunia *et al*., 1998; Gosztonyi & Lundwig, 1995; Ludwig & Bode, 2000; Rott & Becht, 1995). In an inflammatory reaction of the brain, it is universally normal for astrocytes to respond to the inflammatory process irrespective of the aetiology. The primary role of astroctyes is to maintain the CNS microenvironment in order to ensure proper functioning of the neurons (Benveniste, 1992; Eddleston & Mucke, 1993; Schousboe *et al*., 1997). BDV establishes a non-cytolytic chronic infection in primary feline cortical astrocytes causing a severe and specific impairment in their ability to uptake glutamate (Billaud *et al*., 2000). So far, all known BDV isolates are non-cytolytic and highly neurotropic (Lipkin *et al*., 1997; Ludwig & Bode, 2000; Rott & Becht, 1995).

## Viral genome

The BDV genome which is about 8.9 kb is the smallest among all known non-segmented negative-stranded RNA viruses. The genome organisation is characteristic for the order *Mononegavirales*. It has 6 open reading frames (ORF) coding for 6 different proteins, i.e., p40 nucleoprotein, p24 phosphoprotein, GP18 matrix protein, GP94 surface glycoprotein, p190 viral RNA dependent RNA polymerase also called the L polymerase and the 6th protein called X protein or P10 (Briese *et al*., 1994; Cubitt *et al*., 1994).

## Borna disease

BD is an immune-mediated neurological syndrome caused by a BDV. The disease is characterized by meningoencephalitis and usually results in disturbances in movement and behaviour. Natural infection with BDV in animals was originally described in horses and sheep in the southern region of Germany (reviewed by Durrwald & Ludwig, 1997) where the disease is endemic. The horse, donkey and sheep are natural hosts for the virus. There is evidence suggesting that BDV infects a wide variety of animal species and it seems that the disease is more widespread than previously thought (Staeheli *et al*., 2000). The disease has been recognised in cats (Lundgren and Ludwig, 1993; Lundgren *et al*., 1995; Nakamura *et al*., 1996), cattle (Caplazi *et al*., 1994), ostriches (Malkinson *et al*., 1995), and dogs (Weissenbock *et al*., 1998). The virus has also been isolated from a free ranging lynx (*Lynx lynx*) (Degiorgis *et al*., 2000), the first confirmed case of BDV-infection in a large felid. Warmblooded primates are susceptible to experimental infection. The rabbit seems to be the animal of choice for experimental infections. However, other animal species including rats, guinea pigs, chicken, monkeys and tree shrews have been used in experimental studies. The routes of infection for most of the experiments have been intranasal and intracerebral routes because predictable infection could only be achieved via these routes. The clinical features of experimental infections differ greatly from the natural infection. In neonatally infected rats, BDV causes social behavioural changes with no inflammatory response. However, immunocompetent adult rats show similar symptoms as naturally infected animals.

## Pathogenesis

BDV enters the cells of the host by receptor mediated endocytosis. The virus has two important glycoproteins (GP), GP18 and GP94 each having different functions in the life cycle of the virus (Gonzalez *et al*., 1998). GP1 plays a very important role in receptor recognition and virus entry, while GP2 is primarily responsible for fusion event for the release of the virus ribonucleoproteins. BDV has an affinity for neurons and replicates in the neurons. Though the overall mechanism of action is poorly understood, the virus spreads by centrifugal means via the peripheral nerves to other tissues and organs.

## Zoonotic potential

Serological studies suggest that BDV may cause human infection. The relationship between BDV infection and human psychiatric disorders such as schizophrenia, mood disorders and chronic fatigue syndrome have been studied (De la Torre *et al*., 1996). Findings suggest that there might be a link between BDV infection and human mental disorders. Furthermore, BDV has been isolated from the brains of four Japanese schizophrenic patients (Nakamura *et al*., 2000). The virus has also been isolated from the brain of human patients in Germany and USA (Bode *et al*, 1995and 1996). BDV has been used as an experimental model to study abnormal social behaviour.

## Transmission

Neither the mode of transmission nor the reservoir host of the natural infection is known. However, there seems to be a general consensus that the virus is transmitted via the olfactory route as evident by intranasal infection and inflammation and oedema of the olfactory bulb of naturally infected horses (Ludwig *et al*., 1988). Evidence for hematogenous transmission is founded on the basis that viral nucleic acids and proteins have been isolated from peripheral blood mononuclear cells (Rubin *et al*., 1995; Sierra-Honigmann *et al*., 1993). It is also possible for the virus to be transmitted vertically as BDV has been detected in a pregnant mare and the foetus (Hagiwara *et al*., 2000). There are speculations to indicate that vectors or reservoir hosts play a very important role in the transmission of the disease, as experimental infection using a rat model suggests that it might be possible for the virus to be transmitted from an infected host to an uninfected animal via secretions such as saliva, nasal secretions, urine and even faeces (Sauder & Staeheli, 2003). Rodents are proposed as reservoirs for BDV infection because experimental infection of neonatal rats results in viral persistence and the virus has been detectable in the saliva, urine and feaces (Sierra-Honigmann *et al*., 1993). Wild birds are also considered as candidate natural reservoirs for BDV since the virus has been reported in birds excrement (Berg *et al*., 2001). The bicolored white toothed shrew (*Crocidura leucodon*) has been postulated to be one possible reservoir host for BDV. In fact, the brain and the heart of these insectivores have been positive by PCR for BDV (Hilbe *et al*., 2006). It seems there may be more than one BDV reservoir host. Higher incidence of BDV infection is seen mostly in the early spring/summer and this has been attributed to the activities of the reservoir population or to a very long incubation period in the final host.

## Geographical distribution

Borna disease virus infection is endemic in central Europe, i.e., Germany, Switzerland, Austria and Lichtenstein (Caplazi *et al*, 1991). However, the virus causing the disease is thought to be more widespread than previously thought, as evident by recent reports of the disease being diagnosed outside the endemic areas of Central Europe. The infection have been reported in racing horses in Sweden (Berg *et al*., 1999), Japan (Hagiwara *et al*., 1997; Taniyama *et al*., 2001), China (Hagiwara *et al*., 2001), Middle East (Bahmani *et al*., 1996; Yilmaz *et al*., 2002), USA (Kao *et al*., 1993) and several other parts of the world. In addition, the feline strain of Borna disease virus has been isolated in Sweden (Lundgren *et al*., 1995).

## Control, prevention and treatment

### *Strains of BDV*

Essentially, five characterized strains of BDV exist. These are strain V (Genbank accession no U04608), He/80 (L27077), RW98 (AF158629-AF158633), H1766 (AJ311523) and the highly divergent strain No/98 (AJ311524). The two common

laboratory strains are strain V and He/80. Strain V was found in a diseased horse originally from Lower Saxony, Germany, while He/80 also called Herzog/80 was isolated from a horse originating from Baden Wurttemberg, Germany. Strain RW98 was previously described as a possible new human BDV strain. It was isolated from the blood of a psychiatric patient (Planz *et al*., 1999). The highly divergent BDV strain, strain No/98 was isolated from a pony stallion from the federal state of Styria, Austria, where BDV was previously not endemic (Nowotny *et al*., 2000). Strain H1766 was called so because it was isolated from horse no 1766 (Kolodziejek *et al*., 2005).

*Prevention and treatment of BD*

Data collected in endemic areas indicate that the separation of horses and sheep could prevent the outbreak of the disease (Durrwald *et al*., 2006). Sanitary conditions seem to be a critical factor in the spread of the infection. Severe losses had been observed in farms where several animal species had been kept together in one stable. Hence, the recommendation is to improve hygiene and prevent contact between susceptible species. This, however, does not eliminate the disease but contribute to reducing the spread of infection. Due to the high economic losses, BDV vaccines were developed in the 1920s in Germany. The first vaccines used were inactivated brain suspensions of experimentally infected animals. These vaccines did not provide adequate protection in challenge experiments. As a result, two live vaccines were developed. Sequel to World War II and separation of Germany into two countries, a lapinized live attenuated vaccine was developed in Tornau near Dessau, Germany. The Dessau vaccine was based on the strain from a naturally infected sheep.

*Drugs used in the treatment of BD*

Antiviral agent, amantadine sulphate is the drug of choice in the treatment of BDV infection. This drug was used in a patient who had BDV infection as confirmed by tests and also suffered from Parkinson's syndrome with underlying mood disorders. Following treatment, the patient's condition improved and the BDV infection was cleared but the Parkinson's syndrome persisted. The drug has been found to be effective in inhibiting the wild type BDV strains from horses and humans. (Ludwig & Bode, 2000)

## Feline Borna disease or staggering disease

A fatal neurological disorder of cats, called staggering disease, has been linked to BDV infection (Lundgren *et al*., 1995). The infection in cat is characterized by nonsuppurative meningoencephalitis predominantly affecting the brain stem and the limbic system (Lundgren, 1992). Clinically, the disease is characterized by hind limb incoordination and paresis, increased affection, inability to retract the claws and increased appetite. The clinical signs that are observed in staggering disease are a reflection of the multifocal involvement of the nervous system. Lesions in the upper or lower neurons or midbrain are responsible for the hind

limb incoordination and paresis. Increased affection, which is an altered behaviour, may be due to damage to the cortex and limbic system (Oliver & Mayhew, 1987). Some cats that recover from staggering disease sometimes develop an obesity syndrome similar to that seen in rats experimental infected with BDV (Kao *et al*., 1993). Pathological examination of the brain reveals an extensive adventitial mononuclear cuffing, neuronophagia and the presence of inflammatory nodules consisting mainly of macrophages or microglial cells (Lundgren, 1992). This pathological feature is typical of a viral infection. Staggering disease has been observed in Sweden since early 1970s (Kronevi *et al*., 1974).

### Diagnosis

Diagnosis of feline Borna disease in the early days, like any other disease, relied on the conventional method, which was based on clinical signs together with histopathological demonstration of nonsuppurative meningoencephalitis. Histopathology continues to play a very important role in the diagnosis of BDV. It is usually combined with immunohistochemistry for the detection or demonstration of BDV specific antigens (Gosztonyi & Ludwig, 1984). The virus grows in a broad spectrum of cell lines and can therefore be isolated in tissue culture. Due to the fact that BDV is noncytolytic, presence of specific antigens in infected tissue cultures is detected by immunofluorescence and immunocytochemistry (Hirano *et al*., 1983; Pauli *et al*., 1984). With giant strides having been made in molecular biology, polymerase chain reaction (PCR) continues to be a very promising diagnostic tool for BDV. PCR compared to other diagnostic tools is highly sensitive, robust and rapid for detecting the virus (Zimmerman *et al*., 1994).

Infection with BDV either natural or experimental usually elicits a very strong humoral response, and the antibodies that are produced can be detected by indirect immunofluoresence, western blotting or immunoprecipitation. Other methods that can be used for the diagnosis of BDV include ELISA and flow cytometric method for detection of BDV specific antigen in peripheral blood mononuclear cells (PBMC) (Bode *et al*., 1994; Bode *et al*., 1995). However, naturally infected cats seem to develop low or no antibody titres, while experimentally infected cats developed high titres (Johansson *et al*., 2002). Experimentally infected cats had a high titre of antibodies to p40 and p23 and they responded mainly to p40 whereas naturally infected cats had a weak humoral response and although they did respond to p40, the response to p23 was much stronger (Johansson *et al.,* 2002).

## Aims of the project

In broad terms, the aim of the project was to study the molecular epidemiology of Borna disease virus in Sweden. Specifically, the aim was to identify, isolate and subsequently characterize at least one Swedish isolate of BDV. In addition, to use conventional and real-time PCR to detect BDV in clinical and archive samples originating from feline specie. Detection of BDV in clinical samples such as

peripheral blood mononuclear cells would be an important step in allowing institution of early treatment in diseased animals.

For the archive samples, demonstration of BDV by real-time RT-PCR, subsequent sequencing of the PCR products and carrying out a phylogenetic analysis of the sequenced product were the major objectives.

## Results

Archive samples and incoming samples were screened by real-time RT-PCR. Eight samples were positive by real-time RT-PCR. Of these samples, three, O.407/94BG, O.388/96:3 and O.344/94:4 were used for infecting two different cell lines, MDCK and Vero cells, to attempt isolation of the virus by consecutive passaging. Virus was not detected by PCR in infected cells. However, presence of the virus was demonstrated in passages 10-12 in cells infected with the positive control; strain No/98, showing that the methodology used was adequate. Two other samples, O.163/05 and O.69/92:3 with the lowest cycle to threshold value (Ct values) were used to test six sets of primers. The primers used were those described by Kolodziejek *et al*. (2005). Three primer pairs were found to reproducibly amplify C6BDV cells, which are persistently infected with BDV.

## Conclusion

The detection of BDV in eight positive samples from cat by real-time PCR confirmed that the assay is reliable for demonstration of BDV in clinical specimen. Though the virus was not detected upon sub-passages in cells, presence of the virus in later passages in the Vero cell from passage 10-12 in the positive control shows that other factors than the assay may have contributed to the lack of virus isolation. Examples of such factors could be a low amount of virus in the sample that would require more passages than the ones used, or unviable virus in the specimen (though nucleic acid would still be suitable for detection by RT-PCR). The classical PCR for molecular epidemiology needs further optimization as reflected in the fact that only three primer pairs seems to work with the C6BDV cells.

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# Characterization of feline Borna disease virus

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## Abstract

Feline Borna disease, a neurological disease of cats is an infection caused by Borna disease virus (BDV). The infection, first reported in Sweden in 1974, affects other species of animals such as horses in which the disease was first reported. Sheep is the other natural host of the virus, but BDV infection is also seen in cattle, dogs, goats, donkey, and some zoo animals. The disease is widely speculated to be zoonotic as it is believed to affect humans as well. In Sweden, the infection is endemic in central part of Sweden namely in the Stockholm and Uppsala area, where numerous cases have been reported in the past and new cases continue to emerge in recent times. The importance and continued incidence and prevalence of the infection in cat prompted this present study. Archive and incoming samples from cats, horses and dogs were screened by real- time RT-PCR. Eight positive cat samples were detected by real-time RT-PCR. Three of these samples were used for virus isolation and characterization, while two other positive samples were used to test previously described primers. In conclusion, we have been able to detect eight positive samples from the cat by real-time RT-PCR. Though the virus was not detected upon sub-passages in vero cells, demonstration of presence of the virus from passage 10-12 in the positive control show that other factors than the assay may have contributed to the lack of virus isolation The classical PCR for molecular epidemiology needs further optimization as reflected in the fact that only three primer pairs seem to work with the C6BDV used as positive control

## Introduction

In the 18th and 19th centuries in southern Germany, clinical symptoms in horses similar to that of Borna disease (BD) were first described. This was the first record of its kind as regards BDV in any part of the world. There had been an epidemic like outbreak in the 1890 and the first decade of the 20th century in the city of Borna, near Leipzig, Germany, where a large number of horses had died from the disease. The name Bornasche Krankheit (Borna disease) was coined from the district around the city where the epidemic had occurred. In the 1920s, the aetiology of the disease was demonstrated to be a virus (reviewed in Durrwald & Ludwig, 1997; Ludwig & Bode, 2000). As early as the 1950s, feline cases of nonsuppurative meningoencephalitis had been reported in different parts of the world (Hoff & Vandevelde, 1981; Truyen *et al*., 1990). At that time the disease was poorly defined and the aetiology although unknown, was assumed to be of viral origin. However, in 1974, a feline neurological disorder of cat with an unknown aetiology was described in Sweden (Kronevi *et al*., 1974). Cases of feline meningoencephalitis commonly called vingelsjuka in Swedish (staggering disease) continue to occur in mid Sweden even to date. In the 1990s staggering disease was linked to BDV infection (Lundgren *et al*., 1995).

The etiological agent, Borna disease virus, is a single stranded negative sense enveloped non segmented RNA virus (Briese *et al*., 1994; Cubitt *et al*., 1994) and is the prototypic member of the new family *Bornaviridae*, within the order *Mononegavirales* (De la Torre, 1994; Schneemann *et al*., 1995). The virus is believed to be an old evolutionary pathogen that has had time to adapt to a wide range of mammalian hosts. The virus is highly neurotropic having affinity for the limbic system and the reticuloendothelial system.

BDV exhibits several unique features or characteristics, among them are that the genome organisation and nucleotide sequence are typically similar to other members of the order *Mononegavirales*. Unlike other viruses, which replicate and transcribe in the cytoplasm, nuclear localization of replication and transcription is typical of BDV (Pyper *et al*., 1998). The unusually high level of sequence conservation (Formella *et al*., 2000) is unexpected for an RNA virus that has a high rate of replication and transcription and thus likely to mutate often.

The virus seems to have wider host range than previously thought and presumably affects humans as well (Rott *et al*., 1985; Bode *et al*., 1995). The virus causes a persistent infection of the CNS with astrocytes primarily affected (Carbone *et al*., 1989, and 1993). A characteristic feature of the infection is the development of a prominent astrocytosis (Gonzalez *et al*., 1997; Gosztonyi & Ludwig, 1995; Ludwig & Bode, 2000; Rott & Becht, 1995). In an inflammatory reaction of the brain, it is universally normal for astrocytes to respond to the inflammatory process irrespective of the aetiology. The primary role of astrocytes is to maintain the CNS microenvironment in order to ensure proper functioning of the neurons (Benveniste, 1992; Eddleston & Mucke, 1993; Schousboe *et al*., 1997). BDV establishes a non-cytolytic chronic infection in primary feline cortical astrocytes causing a severe and specific impairment in astrocytes ability to uptake glutamate

(Billaud *et al*., 2000). So far, all known BDV isolates are non-cytolytic and highly neurotropic (Lipkin *et al*., 1997; Ludwig & Bode, 2000; Rott & Becht, 1995).

The BDV genome, which is about 8.9 kb, is the smallest among all known negative nonsegemented single-stranded RNA viruses. The genome organisation is characteristic for the order *Mononegavirales*. It has 6 open reading frames coding for 6 different proteins, i.e. p40 nucleoprotein, p24 phosphoprotein, GP18 matrix protein, GP94 surface glycoprotein, p190 viral RNA dependent RNA polymerase also called the L polymerase and the 6th protein called X protein or p10 (Briese *et al*., 1994; Cubitt *et al*., 1994).

In broad terms, the aim of the project was to study the molecular epidemiology of Borna disease virus in Sweden. Specifically, the aim was to identify, isolate and subsequently characterize at least one Swedish isolate of BDV. In addition, to use conventional and real-time PCR to detect BDV in clinical and archive samples originating from feline specie. Detection of BDV in clinical samples such as peripheral blood mononuclear cells would be an important step to allow institution of early treatment in diseased animals.

For the archive samples, demonstration of BDV by real-time RT-PCR, subsequent sequencing of the PCR products and carrying out a phylogenetic analysis of the sequenced product were the major objectives.

## Materials and methods

### *Samples*

Both archive and incoming clinical samples consisting of fifty samples were used for this project. The samples were brain, cerebrospinal fluid and lymphocytes stored at –70°C. Various sections of the brain had been taken from horses, dogs and cats. The sections of the brain taken included cerebellum, cerebrum, medulla oblongata, cortex and hippocampus. Incoming samples were mainly blood samples from horses from the Animal Hospital in Helsingborg, in the south part of Sweden. Lymphocytes were immediately isolated from whole blood samples using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) according the manufacturer's instructions and stored at –70°C for RNA extraction purpose.

*RNA extraction*

Total RNA was extracted with 1000 µl of TRIzol (Invitrogen) for tissue samples and 750 µl for blood samples. Tissue samples were extracted by adding about 25-50mg of tissue into a test tube to which 1000 µl of TRIzol was added. For blood samples, 250 µl of the blood sample was lysed in 750 µl of TRIzol. The samples

were homogenised, incubated for 5 min and 200 µl of chloroform was further added. After mixing vigorously by hand, these were centrifuged at 11,000 × g for 15 min at 4°C. The RNA was transferred to a fresh test tube and 500 µl of isopropyl alcohol was added to precipitate the RNA. Following 10 minute incubation at room temperature, the samples were centrifuged at 11,000 × g for 30 minutes at 4°C. The supernatant was removed and RNA was washed once with 1 ml of 75% ethanol and centrifuged at 7,400 × g for 10 minutes at 4°C. The RNA pellets were air-dried and then resuspended in 25 µl of RNAse-free water. The samples were then diluted at a ratio of 1:5. A 5-µl aliquot was used to measure the RNA concentration and purity using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). RNA was also extracted with RNeasy extraction kits (Qiagen Inc, Valencia, CA, USA) following the manufacturer's instructions.

*Real-time RT-PCR*

A duplex BDV real-time RT-PCR assay was used for screening of the samples (Wensman *et al*, 2004). The reactions were carried out using 2 µl of extracted RNA in a final volume of 25 µl containing 1 × EZ buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM $Mn(OAc)_s$ (Applied Biosystems), 0.08 mg/ml bovine serum albumin, 0.5 mM of dNTPs (GE Healthcare, Uppsala, Sweden), 0.7 µM of each BDV p23 primer, 0.4 µM of each BDV L-gene primer, 0.3 µM of the BDV p23 probe, 0.4 µM of the BDV L-gene probe and 2.5 U of rTth DNA polymerase (Applied Biosystems). The reactions were run in a Rotor-Gene 3000 (Corbett Research, Australia). The RT reaction was performed with an initial incubation of 42°C for 5 min, followed by incubation at 60°C for 20 min. These were then processed through 45 cycles of 5 s at 95°C, 30 s at 50°C. The data were analysed and presented with Rotor-Gene Real-Time Analysis Software 6.0 (Build 38) (Corbett Research). For primer and probe sequences, see Wensman *et al* (2004).

*Classical RT-PCR with Phusion DNA polymerase*

cDNA synthesis

The RT reaction was performed in a final volume of 40 µl containing 500 ng of total RNA. The RNA was mixed with 1.06 µg of random hexamers and 1 mM of dNTPs and incubated at 65°C for 5 min. Then a reaction mix containing 1 × first strand buffer, 10 mM DTT and 24 U of RNAse out (Invitrogen) and 200 U of reverse transcriptase, Superscript II (Invitrogen) was added.

The RT reaction was performed at 42°C for 50 minutes. To inactivate the enzymes samples were incubated at 70°C for 15 minutes.

The six sets of primers used for PCR were those described by Kolodziejek *et al*. (2005) and Berg *et al.* (2001) (see Tables 1 and 2). RNA samples from the two cats with the lowest cycles to threshold (Ct) values that had been positive by real-time RT-PCR were reverse transcribed and their cDNA were used to test the primers. Also, RNA extracted from the C6BDV cell line, persistently infected with BDV was used as a control.

The PCR was performed by mixing 1µl of cDNA with 0.4 µM of each forward and reverse primer, 1 × Phusion buffer (Finnzymes OY, Espoo, Finland), 250 µM of each dNTPs, 0.5 U of Phusion DNA polymerase (Finnzymes OY) and DMPC-treated water to a total volume of 25 µl. The cycling conditions were as follows: An initial denaturation step of 98°C for 30 s followed by 40 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 5 min. The PCR products were run on 1.5% agarose gels in 0.5 × TBE buffer for 1 h at 110V, stained with ethidium bromide and then visualized on a transiluminator. The size of the PCR product was determined by comparing to a 100-bp DNA ladder.

Table 1.Primer pairs used for the PCR and expected size of the PCR products.

| Primer pair | Forward primer | Reversed primer | PCR product size (bp) |
|---|---|---|---|
| 1 | BDV 1F | BDV 625r | 652 |
| 2 | BDV 218F | BDV796r | 579 |
| 3 | BDV587F | BDV 1161r | 575 |
| 4 | BDV 778F | BDV1518r | 741 |
| 5 | BDV1327F | BDV1837r | 511 |
| 6 | BDV1695F | BDV2138r | 444 |

There were two non-template controls (NTC) for each reaction. In order to increase the sensitivity a nested RT-PCR assay, combining the BDV1f and p23B primers as the outer primer pair, was used. One microlitre of PCR product was taken from the first round of PCR and used as a template for the next round of PCR. PCR product from the first PCR reaction was diluted in the ratio of 1:10, and the rest was then purified using Wizard® SV Gel and PCR clean-up system (Cat # A9282 Promega). The diluted and purified PCR products were also used as templates for the 2nd round of PCR. The same procedure was applied to P40A and P40B. The thermal profile for P23A and P23B, P40A and P40B were initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing temperature of 55°C for 30 s, and extension at 72°C for 2 min and a final extension at 72°C for 5 min. For P40C and P40D, P23C and P23D the thermal profile were an initial denaturation at 95°C for 1 min, followed by 35cycles of denaturation at 95°C 30 s, an annealing temperature 60°C for 30 s, and extension at 72°C for 30 s and a final extension at 72°C for 5 min.

*Characterization of virus isolates*

Three RNA samples from cats that had been positive for the BDV by real-time reverse transcriptase PCR were used for virus infection and subsequent passaging in two different cell lines, MDCK and Vero cells.

The cells were sub-cultured up to the 12th passage and at each passage cells were frozen down for RNA extraction, for later passaging and also lysed for preparation of protein extracts to check for viral proteins.
MDCK and Vero cells were grown in (media) supplemented with 10% FCS, L-glutamine, and containing a mixture of penicillin and streptomycin as antibiotic. The brain samples of the three samples from the cats that had been positive by real-time RT-PCR: O.407/94 bg, O.388/96:3 and O.344/96:4 were thoroughly homogenized using a plastic pestle. Further homogenization was done by pipe tting up and down several times, then 5 ml of cell culture medium was added. The infection was done by transferring 100 µl of brain suspension onto semi-confluent MDCK and Vero cells, grown in 25 $cm^2$ tissue culture flasks. After 1 hours of incubation, the cells were washed twice in PBS and fresh cell culture medium was added. Subsequently, the cells were sub-cultured every third-fourth day. For the positive control, 100 µl of Vero cells infected with the No/98 strain was used to infect the cells following a similar procedure. The infection of the positive control was done after decontaminating the hood. For each of the cell lines, there were two controls, one positive control, strain No/98 and a negative control which was uninfected cell line, i.e., uninfected Vero cells and uninfected MDCK cells.
The cells were sub-cultured up to the 12th passage and at each passage cells were frozen down for RNA extraction, for later passaging and also lysed for preparation of protein extracts to check for viral proteins.
In parallel, uninfected MDCK and Vero cells of the same passage level as used for infection were sub-cultured and processed the same way as the infected cells.

## Results

*BDV real-time RT-PCR screening*

First, archived samples from cats, dogs and horses were screened using the BDV real-time RT-PCR assay. Following the extraction of RNA and the detection of the Borna disease virus gene by duplex real-time RT-PCR, 8 samples were positive for the P23 region (Fig 1a). The RNA of these samples was reverse transcribed into their complementary DNA (cDNA).

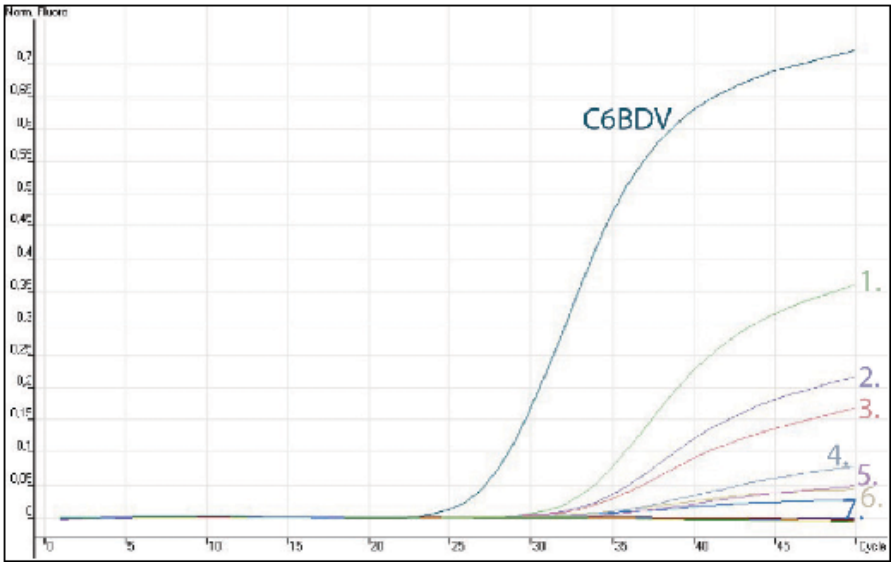


*Fig. 1a.* Real-time RT-PCR detection of Borna disease virus P23-gene. 1, O.338/94:4; 2, O.388/96:3; 3, O.407/94BG; 4, O.388/96:1; 5, O.334/96:2; 6, 334/96:5; 7, 4135.

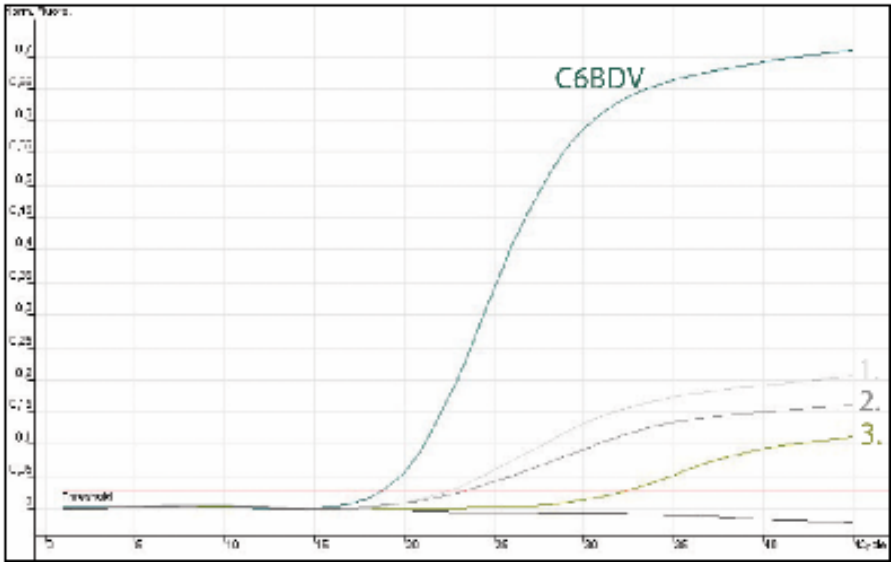


*Fig. 1b.* Demonstration of BDV by real-time RT-PCR. Two samples with low Ct values were used to test primers for generation of PCR products for molecular epidemiology: (1, O.69/92:3; 2, O.407/94BG; 3, O.163/05; 4, O.388/96:1).

Two samples, O.163/05 and O.69/92:3, with the lowest cycles to threshold values (Ct values) were used for testing the primers (Fig 1b), while three samples

O.407/94 BG, O.388/96:3, O.344/96:4 were used for infecting the two cell lines, MDCK and Vero cells (Fig. 1a).

### *Optimisation of classical PCRs for molecular epidemiology*

For molecular epidemiology, primers described in Kolodziejek *et al*. (2005) and Berg *et al*. (2001) were used. The two feline samples with the lowest threshold Ct values, indicating the highest presence of viral RNA, were used in an initial test (Fig 2). PCR products were only seen clearly using primer pairs 3, while Primer pair 4 and 6 produced faint bands and in one case (primer pair 3) the size was larger than expected.

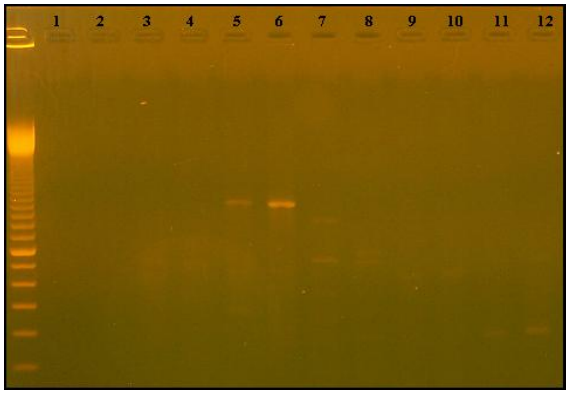


*Fig. 2*. Testing of the primers. Lanes 1 and 2: primer pair 1; lanes 3 and 4:: primer pair 2; lanes 5 and 6: primer pair 3: lanes 7 and 8: primer pair 4; lanes 9 and 10:primer pair 5; lanes 11 and 12: primer pair 6 (as listed in Table 1).

The primers were originally designed to fit the BDV strain V. Therefore, the C6BDV cells infected with BDV He/80, which has a similar sequence as strain V was used to further test the primers. This test showed that only the three first primer pairs were working optimally (see Fig 3). Our observations revealed that the forward primers seem to be working at least for the first three pairs. We then decided to use the forward primer BDV587F to test if the reverse primers were working. Also included in this test was forward primer, BDV778, which seem to work. It was combined with BDV1161r the last reverse primer, which seem to work. This primer pair combination was the only one that seems to work producing PCR product of the expected size. In this experiment primer pair 3 was used as a control (Fig 4).

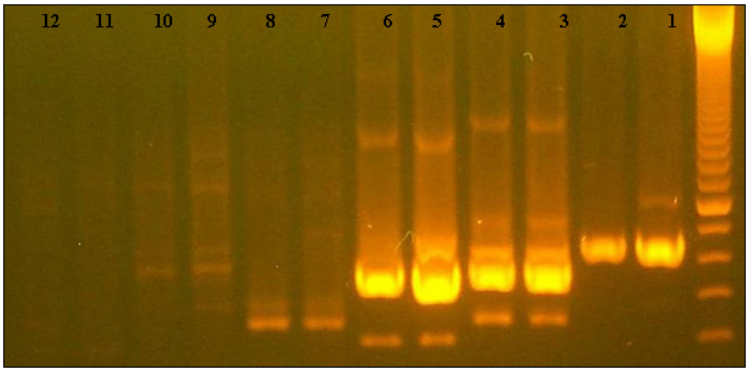


*Fig. 3*. Testing of the primers with the C6BDV cells and two samples with the lowest ct value. Lanes 1, 2, 7 and 8: primer pair 1; lanes 3, 4, 9 and 10: primer pair 2; lanes 5, 6, 11 and 10: primer pair 3 (as listed in Table 1).

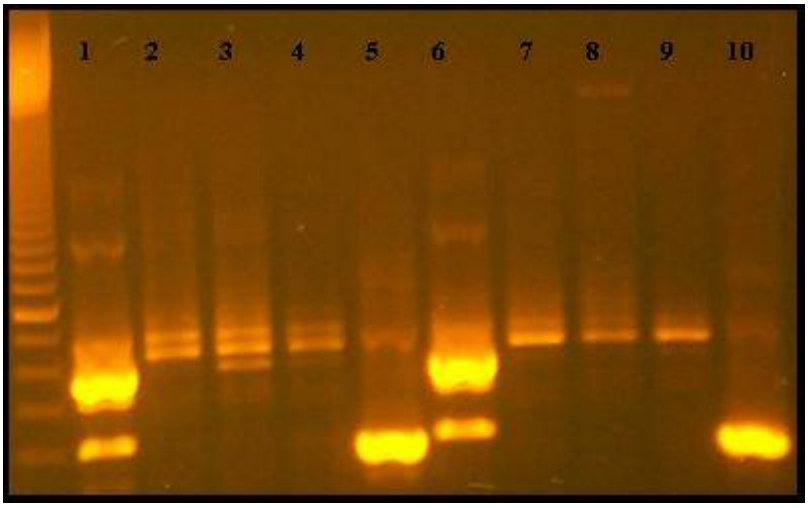


*Fig. 4*. Testing of the reverse primers. Lane 1 and 6, DV587 & BDV1161 (control); Lane 2 and 7, BDV587 and 1518r; Lane 3 and 8, BDV 587 and 1837r; Lane 4 and 9, BDV 587 and 2138r; Lane 5 and 10, BDV778 and 1161r.

### *Characterization of virus isolates*

Brain suspensions of three naturally infected cats positive by real-time RT-PCR were transferred to MDCK and Vero cells in order to try to isolate the virus. The BDV strain No/98 was used as a positive control and uninfected cells treated the same way as the infected was used as negative controls.

RNA materials frozen down from passage 1 to 12 for the uninfected and infected cell lines, MDCK and Vero cells were subjected to real-time RT-PCR. C6BDV cells were also used along with strain No/98 as positive controls. In each PCR, there were two non-template controls (water). BDV could not be detected in any of the uninfected and infected cell lines but virus was demonstrated in later passages in the positive control, strain No/98 (P10, P11, and P12) (data not shown 5).

## Discussion and conclusion

Compared to the positive control, C6BDV cells, the eight samples that were positive by real-time RT-PCR had high cycle threshold values. This probably might be an indication that the samples contain small amount of viral load in the first instance.

Our inability to detect BDV by real-time RT-PCR from RNA materials frozen down from various cell passages might be due to small amount of viral load in the samples, in spite of the fact that the three samples that were used for infecting the different cell lines had the lowest Ct values. Other possible explanations for the failure to demonstrate BDV gene in the infected cell lines could be due to the fact that the three samples that were used for the infection were obtained from the brain and as we know the brain contains a lot of cellular debris such as fat and some other toxic materials and other contaminants. These contaminants might have inhibited the PCR reaction. Perhaps, it is the same line of thought that influences the use of lymphocytes rather than blood sample for diagnosis of BDV. This fact might also have led to the purification of the Japanese human brain samples first by ultracentrifugation to get the ribonucleoproteins before extracting the isolates (Nakamura *et al*., 2000). Cellular debris from the brain might have affected the cells and that could be a reason for not detecting the virus. BDV grows in several established cell lines but the feline strain has been demonstrated to grow only in embryonic mink brain cells. The virus was detected in the third passage but later disappeared (Lundgren *et al.*, 1995). It is not known if the virus grows in other cell lines, which we did not test or use.

However, BDV was demonstrated though in the later passage of the positive control, strain No/98, which is an indication that our systems do work. Strain No/98 is however a cleaner material compared to the brain suspension from the cat.

The unspecific banding that was observed on the gel electrophoresis following PCR with cDNA from feline virus might be due to primer mismatches. The primers had been designed based on the nucleotide sequence of strain V. This was somewhat confirmed when the primers were tested using the C6BDV cells, persistently infected with a strain similar in sequence with the V strain. The banding was more specific and the PCR product corresponded to the expected sizes.

Characterization of a feline strain of BDV would enable comparison with other strains that have been sequenced particularly from the horse, possibly emphasising the similarities and differences. It might also be important in tracing the origin of the infection. The development of a good PCR for diagnosis is important particularly in intra vitam diagnosis of the disease. The PCR system in place should be one that can detect all possible strains of BDV from different species of animals. Perhaps the incidence and prevalence of BDV in general and feline BDV

in particular has been underestimated due partly to the fact that the PCR systems currently in use has failed to detect the BDV, as a result of the numerous problems associated with the disease.

It would be interesting and of scientific benefit to characterize a feline strain of BDV isolate as this would aid in our understanding of the pathogenesis, epidemiology, diagnosis, treatment and possibly vaccine development in the nearest future.

In conclusion, we have by real-time RT-PCR been able to detect eight positive samples from the cat. Though we were unable to isolate the virus in later passages in the Vero cells, we did demonstrate the virus from 10-12 passages of the strain No/98, the positive control. The classical PCR for molecular epidemiology needs further optimization as reflected in the fact that only three primer pairs seem to work with the C6BDV cells.

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