



Interferon expression in feline Borna disease



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Photo: Anna-Lena Berg, Astrazeneca.

Summary

Borna disease is caused by Borna disease virus (BDV). This disease is characterised as a neurological illness of the central nervous system (CNS), known to occur in several vertebrates like horses, cats and sheep.

Cats mainly at the age of 1 to 4 years, living outdoors, have also been shown to contract the BDV infection. A typical sign in cats of BDV infection is a change in behaviour, i.e. some cats get increased affection and meow more than usually. In addition, a lot of cats get ataxic and their balance is adversely affected. The route of infection is still not fully known. One theory is that the virus spreads and infects through saliva and the respiratory tract, respectively. Some studies have shown that the infection is not commonly transmitted between cats. Thus, it seems more likely that another species, such as rodents or birds, are the carrier of the disease. This hypothesis seems more likely since the cats that have been diagnosed as carriers of BDV have been living outdoors. There are no sufficient diagnostic methods for BDV. Therefore necropsy and histopathologic examination of the brain tissue is the method for diagnosis and confirmation of BDV infection.

The aim of this study was to investigate whether the gene expression, measured by quantitative RT-PCR, of IFN- α , - β and - γ in the brain tissues of Borna disease diagnosed cats is induced. To normalize the IFN gene expression, the reference genes, HPRT, RPS7 and YWHAZ, were used. In addition, PCR-products were confirmed by gel electrophoresis. With the use of different PCR measurements for the expression of reference genes it was studied whether Borna disease was associated with gene expression of cytokines in the brain.

When comparing the C_T -values of IFN- γ with the histopathological finding it was clearly evident that Borna disease diagnosed cats had a much higher gene expression than the other cats. However, a majority of non virus diagnosed cats showed higher C_T -values or no C_T -values at all. Consequently, results showed clear association between Borna disease diagnosis and increased IFN- γ gene expression.

Sammanfattning

Vingelsjuka hos katt orsakas av bornavirus (BDV), som även orsakar neurologisk sjukdom hos bl.a. får och häst.

Vad man vet så drabbas främst utomhuskatter, i åldern 1 till 4 år, av bornavirus. Vanliga symtom som förekommer hos katter med bornavirusinfektion är förändringar i beteendet. Vissa katter blir mer sociala och jamar mer än vanligt, ibland förekommer det även att de blir aggressiva. Ett annat typiskt symtom är förlamning i bakbenen och att de tappar balansen. Idag vet man inte säkert hur viruset sprider sig. En teori är att viruset sprider sig och infekterar genom saliv och luftvägarna. Studier har visat att viruset vanligtvis inte smittar katter emellan. Det verkar mer troligt att smittobäraren är av en annan art som t.ex. gnagare eller fåglar. Dessa hypoteser verkar mer troliga eftersom de katter som blivit diagnostiserade med bornavirus vistas utomhus. Idag finns det inte tillräckligt bra diagnostiska tester för BDV. Därför används obduktion och histopatologiska undersökningar av hjärnvävnaden för att diagnostisera och fastställa om det är en bornavirusinfektion.

Syftet med denna studie var att undersöka genuttrycket av IFN- α , - β och - γ i hjärnvävnaden hos vingelsjuka katter. För att normalisera variationen i genuttrycket användes referensgenerna HPRT, RPS7 och YWHAZ. Gelelektrofores användes sedan för att påvisa PCR-produkter. Genom att använda sig av olika PCR-mätningar för uttrycket av referensgenerna så studerades det om vingelsjuka associerade med genuttrycket av cytokiner i hjärnan.

När C_T -värdena från IFN- γ och de histopatologiska undersökningarna jämfördes kunde man konstatera att de katter som diagnostiserats med vingelsjuka hade ett mycket högre genuttryck än de andra katterna. Hursomhelst, majoriteten av icke-diagnostiserade vingelsjuka katter visade högre eller inga C_T -värden överhuvudtaget. Resultaten visade klara samband mellan vingelsjuka katter och ökat IFN- γ uttryck.

Introduction

Borna disease is caused by Borna disease virus (BDV). This disease is characterised as a neurological illness of the central nervous system (CNS), known to occur in several vertebrates like horses, cats and primates (Ludwig & Bode, 2000). In the late 19th century, after an illness breakout in horses in the city of Borna in South-east Germany the disease got its name Borna disease (BD) (Staeheli *et al.*, 2000). In 1920, it was for the first time possible to isolate BDV and since then this virus has been reported to infect different species from all continents, except Africa. After those discoveries new animal species have been shown to be susceptible to the virus, for example it was recently reported from Finland that rodents proved to have antibodies towards BDV (Kinnunen *et al.*, 2007).

Cats at the age of 1 to 4 years, living outdoors, have also been shown to subtract the BDV infection. A typical sign of BDV infection in cats is a change in behaviour, i.e., some cats get more affective and meow more than usually. In addition, a lot of cats get ataxic and their balance is adversely affected (Berg *et al.*, 1998). These signs usually start appearing between December and May. In Sweden most cases have been found in Mälardalen. The virus causes a non-cytolytic, persistent infection and has been shown to enhance viral gene expression in the limbic structures. Moreover, the amount of virus has been shown to increase in neuroglial cells and to cause neuronal structural alterations within the hippocampal formation (Hans *et al.*, 2004).

The route of infection is still not fully known (Kamhieh & Flower, 2006). One theory is that the virus spreads and infects through saliva and the respiratory tract, respectively. Some studies have shown that the infection is not commonly transmitted between cats. Thus, it seems more likely that another species, such as rodents or birds, is the carrier of the disease. This hypothesis seems more likely since the cats that have been diagnosed as carriers of BDV have access to outdoors (Berg, *et al.* 1998). There are no sufficient diagnostic methods for BDV. Therefore necropsy and histopathologic examination of the brain tissue are the methods for diagnosis and confirmation of BDV infection.

Today there is however new diagnostic tools on the way that are based on molecular techniques, i.e. the PCR technique. By the sensitive PCR method the negative single-stranded RNA of the Borna virus can be amplified and detected. This is also a highly specific diagnostic tool for detection of Borna virus because it is presently the only known member of the *Bornaviridae* family that is present in Sweden (Degiorgis *et al.*, 2000). Besides specific viral detection by PCR it is also possible to use PCR for gene expression analysis of important immune system markers of infection and inflammation processes, such as the cytokines (Staeheli *et al.*, 2000). The aim of the present study was to use PCR for detection of gene expression of a selection of cytokines and relate these results to histopathological alterations in the brain of Borna virus diagnosed cats.

The PCR technique can be used to detect cytokine gene expression because it is one of the most sensitive methods for the detection of the low amount of mRNA that usually is obtained when there is a limited amount of tissue available, such as in parts of the brain tissue (Pfaffl, 2001). When using the PCR technique RNA has to be transformed to complimentary DNA (cDNA), with the help of the enzyme reverse transcriptase. This transformation is needed because the cDNA is a much more stable molecule than RNA and it can tolerate the high

temperatures that are needed in the PCR. Even though cDNA has a different structure composed to RNA it contains the same information. The PCR technique can thus be used to measure the actual level of RNA in the test sample. During the real-time RT-PCR C_T -values are measured, which is a measure of how many cycles it takes before the fluorescence of a sample to reach a specific threshold. Consequently, C_T -values reflect the amount of RNA in the test sample. The C_T -value decreases when the mRNA increases (Livak *et al.*, 2001).

The reference genes in the PCR reactions were HPRT, RPS7, YWHAZ, RPL30, RPL17, and GAPDH. These reference genes were used as a qualitative and quantitative measure in relation to the variation in gene expression obtained in the cytokine PCR reactions. Thus, reference genes were used as a control, and should be similarly expressed in all studied cells (Penning *et al.*, 2007).

The present study was focused on a selected number of cytokines, i.e., IFN- α , IFN- β and IFN- γ . IFN- α is known to be associated with neurotoxicity. Thus, a high gene expression in the brain indicates processes that are associated with immune activation and potential adverse effects. Even though IFN- α and - β are structurally different they bind to the same cell surface receptor and provoke similar biologic responses, activation of immune cells, such as natural killer cells (NK) and macrophages. IFN- α and - β synthesis are stimulated during viral infections, especially when double-stranded RNA is produced by viruses during their replication in infected cells. IFN- γ is an essential cytokine in both the innate and the adaptive immune system, and it is known to be involved in the anti-viral response. IFN- γ acts on B cells thereby inducing antibody responses that participate in phagocyte-mediated elimination of microbes. IFN- γ also activates neutrophils and stimulates the activity of NK cells which are able to kill virus infected host cells (Abbas *et al.*, 2003).

Aims of the project

The aim of this study was to investigate whether the gene expression of IFN- α , - β and - γ in the brain tissues of Borna disease diagnosed cats were induced. To normalize the gene expression, reference genes HPRT, RPS7, YWHAZ, RPL30, RPL17 and GAPDH were used. With the use of different PCR measurements for the expression of reference genes it was studied whether feline Borna disease was associated with gene expression of cytokines in the brain.

Material and methods

Animals

Cats with and without clinical signs of feline Borna disease (staggering disease) were used in this study. All cats were euthanized and necropsied at SLU or SVA. Tissue samples were either frozen directly at -70°C or first stored in RNA later (Ambion) for 1-7 days at 4°C and subsequently stored at -70°C . For more information about the cats, see Table 1.

Table 1. Table showing histological finding and IFN- γ , $-\alpha$, and $-\beta$ expression of the cats, as well as BDV RT-PCR results.

Autopsy nr	Histopathological finding	BDV	IFN- γ	IFN- α	IFN- β	
O.98/05	multicentric malign lymphoma	negative		0	0.51	0.4
O.39/05	hemorrhagic and erosive cystitis	negative		0	0.53	0.47
290/06	multicentric malign lymphoma		negative	0.23	0.35	0.49
O.38/05	not determined	negative		0	0.54	0.48
067-07	negative section	negative		0	0.4	0.42
07/610	non-supp meningoencephalomyelitis	negative		0.47	0.45	0.55
0,2246/08						
Cortex						
cerebrin	non-supp encephalomyelitis	negative		0.39	0.44	0.57
0,2246/08						
Meseceph						
alon	non-supp encephalomyelitis	negative		0.43	0.57	0.6
0242/05		negative				
Basala						
delar	non-supp meningoencephalomyelitis			0.4	0.46	0.57
0234/05						
Corebra	non-supp meningoencephalomyelitis	negative		0.5	0.51	0.55
0,1202/05						
Cortex						
cerebri	not determined	positive		0	0.42	0.4
0231/05						
Basala						
delar		negative		0.43	0.5	0.57
01397/04						
M.spinal	acute non-supp meningoencephalomyelitis	negative		0.42	0.45	0.5
0,111/03						
Cerebrum	non-supp meningoencephalomyelitis	negative		0.27	0.47	0.57
0,252/03						
Cortex						
cerebrum	non-supp meningoencephalomyelitis	negative		0.49	0.54	0.57
0,828/03						
Cortex	non-supp meningoencephalomyelitis	negative		0.32	0.47	0.52
0,1405/05		negative				
M. spinal	meningioma			0.42	0.47	0.54
0,795-04						
Basala						
delar	acute non-supp meningoencephalitis	negative		0.49	0.47	0.56
0,19/04						
Cerebrum	non-supp meningoencephalomyelitis	negative		0.39	0.45	0.46
0,469/03						
Cortex						
cerebri	non-supp meningoencephalomyelitis	negative		0.47	0.49	0.55
0199/09						
Cortex		negative		0.22	0.37	0.42
0171/09						
Mesencep						
halon	pulmonary adenocarcinoma	negative		0	0.47	0.45
0180/09						
Ryggmärg	Non-purulent meningoencefalomyelit	positive		0.47	0.51	0.57
0154/09						
Cortex	not determined	negative		0	0.36	0.39
0172/09						
Cortex	metastasizing epithelial carcinoma larynx	negative		0.26	0.4	0.38

RNA extraction of brain tissue with on-column DNase treatment.

The purpose of this method was to extract RNA from cat brain tissue of healthy cats and cats infected with Borna virus. Tissue samples from the brain of sick and healthy cats were taken at pathological examination and RNA was thereafter extracted from brain tissue samples. In addition, a brain tissue sample from a horse was included as an extra control. A cleaning step to get rid of impurities was also performed. After this step pure RNA was transformed to cDNA that could be analysed by the PCR method.

The brain tissue from the -80°C freezer was taken out, and with a surgical blade and plastic plates a tissue sample from the brain was cut and transferred into an eppendorf tube containing 250 µl of Qiazol (Qiagen). After homogenisation 500µl Qiazol was added before the eppendorf tube was incubated at room temperature for 5 min. After the incubation time 150µl chloroform was added to the tube and placed in the centrifuge at 12000 g for 15 min at 4°C.

During the centrifugation the samples were separated into three layers. The pink layer at the bottom of the tube contains protein, the white middle layer contains DNA and the transparent layer on the top contains RNA. To separate the RNA layer from the other layers a pipette was used to transfer the small volumes of the RNA into a new tube. The same estimated volume of 70% ethanol as RNA was added into the tube with RNA and mixed thoroughly by vortexing. The solution was pipetted into an RNeasy Spin Column (Qiagen) and centrifugated at ≤8000g for 30 seconds at room temperature. The flow-through was then discarded. 350µl Buffer RW1 was added to the column and the samples centrifuged once again at ≤8000g for 30 seconds. The flow-through was discarded.

A reaction mix containing, 10µl DNase I stock solution and 70µl Buffer RDD (Qiagen) was prepared and mixed. This solution was transferred to the RNeasy spin column membrane and incubated at room temperature for 15 minutes. After the incubation period an additional 350µl Buffer RW1 was added to the column and the sample centrifuged. The flow-through was discarded. 500µl Buffer RPE was transferred onto the RNeasy Spin Column and centrifuged again. The flow-through was discarded. Another 500µl Buffer RPE was added, but this time the centrifugation time was 2 minutes in order to dry the silica-gel. After this step the RNeasy Spin column was placed into a new collection tube and centrifuged at full speed for 1 minute.

To elute the RNA from the membrane in the column it was transferred into a new collecting tube. RNA was eluted by 30µl RNase-free water and placed onto the silica-gel and centrifuged for 1 minute at ≤8000g. This step was repeated once more in order to increase the yield of RNA.

Eppendorf tubes were marked and 15µl from the elution step was transferred in each of the tubes which were kept at -80°C until further analysed.

To measure the RNA concentration and also to check the purity of the extracted RNA (260/280) NanoDrop was used.

cDNA synthesis

The purpose of this step was to translate RNA to cDNA, because the cDNA is much more stable than the RNA molecule.

Two eppendorf tubes were marked Mix 1 and Mix 2. Mix 1, contained 2.5µl OligodT (Invitrogen), 2.5µl dNTP (10mM), and Mix 2 10µl 5x first strand buffer, 2.5µl 0.1 M DTT and 2.5µl RNase out. The RNase out was the last solution that was added in Mix 2. Mix 1 and 2 were kept on ice until they were needed.

One PCR- tube was marked (+RT) before 5µl of Mix 1, and 28µl of 625 ng RNA was added into the tube. The tube was placed in the mini cycler at 65°C for 5 minutes. When the temperature had decreased to 4°C 15µl of Mix 2 was transferred into the +RT tube. After that 10µl from the +RT tube was transferred into a new tube marked –RT. 2µl Superscript III was transferred into the +RT tube. After this step both the +RT and the –RT tubes were placed in the mini cycler. The RT reaction started at 25°C for 5 min before the temperature was increased to 50°C for 30 minutes. After that the temperature was increased to 55°C for another 30 minutes. To inactivate the RT-enzyme, the samples were finally incubated at 70°C for 15 minutes. To keep them safe the tubes were kept in the -20°C freezer.

Quantitative PCR (qPCR) of reference genes

The rRT-PCR (real time reverse transcription polymerase chain reaction) is used for measurements of expression of different genes. The selected reference genes in the present study were HPRT, YWHAZ, RPS7, RPL17, RPL30 and GAPDH. The SYBR green-PCR differs from the regular RT-PCR in that it binds to double-stranded DNA with a fluorescing molecule resulting in measurable colour reaction. When the SYBR green molecule is not bound it does not emit any fluorescence. However, when it connects with the double-stranded DNA it becomes fluorescent and is thereby possible to detect.

Both TaqMan and SYBR-green real-time PCR are methods that are used in the technique real-time PCR and permit the detection of PCR products using a fluorescent signal. The difference between these methods is that TaqMan real time-PCR contains a TaqMan probe, which is an oligonucleotide, specific for the target sequence DNA, and SYBR-green PCR contains a flourogenic dye that emits a strong fluorescent signal when binding to double-stranded DNA. This techniques can be used for quantification of different RNAs, including cytokine mRNA.

SYBR-green qPCR

12.5µl Power SYBR green MM, 0.5µl DMPC water, 1µl F-primer (10µM) and 1µl R-primer (10µM) were blended in one eppendorf tube. 15µl of this master mix and 10µl of the diluted cDNA (1:10) were transferred into a PCR tube. This tube was then placed in the PCR machine. The program was started at 95°C for 10 minutes. When the cycling step began the temperature was 95°C for 15 seconds. After this step the temperature was decreased to the annealing temperature for the specific reference gene which varies (Table 2). This cycling step was repeated 40 times before the holding temperature was set at 60°C for 30 seconds. The program was ended by a melt curve analysis at 60-99°C. This experiment was duplicated for all genes. For primer sequences, see Table 2.

Table 2. All reference genes that were used during SYBR-green PCR.

Primer	Forward primer (5' → 3')	Reverse primer (5' → 3')	Tm (°C)	Reference
RPL17	CTCTGGTCATTGAGCA CATCC	TCAATGTGGCAG GGAGAGC	58	L.C. Penning (2007)
RPL30	CCTCGGCAGATAAATT GGACTGTC	TGATGGCCCTCTG GAATTTGAC	64	L.C. Penning (2007)
HPRT	ACTGTAATGACCAGTC AACAGGGG	TGTATCCAACACT TCGAGGAGTCC	60	L.C. Penning (2007)
RPS7	GTCCCAGAAGCCGCAC TTTGAC	CTCTTGCCCACAA TCTCGCTCG	69	L.C. Penning (2007)
YWHAZ	GAAGAGTCCTACAAA GACAGCACGC	AATTTTCCCCTCC TTCTCCTGC	65	L.C. Penning (2007)

TaqMan qPCR

The PCR mixture containing QuantiTect PCR MM, probe (0.2 μM), forward primer (0.4 μM) and reverse primer (0.4 μM) was prepared. 15 μl of the master mix and 10 μl of the diluted cDNA (1:10) were transferred into a PCR tube. This tube was then placed in the PCR machine. The program was started at 95°C for 15 minutes. When the cycling step began the temperature was 95°C for 15 seconds. After this step the temperature was decreased to the annealing temperature at 60°C in 30 seconds. For primer and probe sequences, see Table 3.

Table 3. Primers and probes that were used during TaqMan PCR.

Cytokine	Primer	Sequence (5' -3')	Probe	Probe sequence (5' -3')	Reference
IFN-α	fIFNa.112f	CTTGACGCTCCTGGACAAA	fIFNa.141p	TCCCTGCCATCTCCTGFCAGAAAGG	Foley et al. 2003
	fIFNa.212r	ACTGGTCTCCACCAGAACACG			
IFN-β	fIFNb.290f	GCACGGATGGAATGAGACC		FAM-ACCTCCTTGCACACTCCAATGGC-BHQ	J Wensman & M Berg unpublist
	fIFNb.427r	T CAGGT T CAGAAGGTCGTATTG			
IFN-γ No.1	IFN.433f	CACCAAGATCTAACCTGAGGAAGC	IFN.378p	CAGATCATCCACCGAATTTGAATCAGC	Leutenegger et al. 1999
	IFN.524r	TATTGCAGCAGGATGACCAT			
IFN-γ No.2	IFN.141F	TGGTGGTTCGCTTTTCGTAG	IFN.152p	CATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAAT	Leutenegger et al. 1999
	IFN.225r	GAAGGAGACAATTTGGCTTTGAA			
Feline GAPDH	GAPDH.57f	GCCGTGGAATTTGCCGT	GAPDH.77p	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA	Leutenegger et al. 1999
	GAPDH.138r	GCCATCAATGACCCCTTCAT			
BDV	p-flap fwd	AATAAATCATAAGAACCCTCCATGATCTCAGAY	BDV P probe	FAM-CAGCGAACCAGGAGGAGCAGCTATC-BHQ	Wensman et al. 2007
	p-flap bwd	AATAAATCATAACTCYGTCACTAGCTTCTTGATRAG			Afonina et al. 2007

IFN (real-time) qPCR

In this part of the experiment changes in the studied cytokine genes, i.e., IFN-α, -β and γ were controlled with real-time PCR.

As a first step 12.5 μl QuantiTect PCR MM, 1 μl F-primer (10 μM), 1 μl R-primer (10 μM) and 0.5 μl Probe were blended in an eppendorf tube. Using RT-tubes (Corbett Research) specific for the PCR machine, 15 μl of the master mix and 10 μl of cDNA diluted 1:10 were transferred to each of the tubes after which the tubes were capped.

The PCR tubes were transferred into the real-time PCR machine (Rotorgene 3000, Corbett Research). To activate the enzyme, the samples were incubated at 95°C for 15 minutes. The cycling started at 94°C for 15 seconds with an annealing temperature of 60°C for 30 seconds. The cycles were repeated 45 times.

This experiment was duplicated for all genes. For primer and probe sequences, see Table 3.

Gel electrophoresis

Gel electrophoresis is normally performed after running real-time PCR for confirmation of products. This step is however not absolutely necessary when using TaqMan PCR. However, TaqMan PCR is a more sensitive and specific PCR analysis than detecting amplicons by agarose gel electrophoresis because it uses a fluorescent tag probe for detection of the amplicons (Chan *et al.*, 2002).

The right product in the sample or contamination can be detected by gel electrophoresis. The size of the product was in the present study confirmed by using a ladder of 100 bp. In initial evaluation of PCR system, the size of PCR product were confirmed by gel electrophoresis.

The agarose gel was made by 250 ml 1.5% agarose gel; i.e. 3.75 gram agarose was dissolved in 250 ml TBE. The solution was prepared in a microwave oven.

BDV-PCR

As a first step 4.25 μ l DMPC-H₂O, 12.5 μ l QuantiTect PCR mix, 2 μ l BSA (1mg/ml), 1.75 μ l F-primer P-flap (10 μ M), 1.75 μ l R-primer P-flap (10 μ M) and P23-probe (10 μ M) were blended in an eppendorf tube (Wensman *et al.*, 2007). Using RT-tubes (Corbett Research) specific for the PCR machine, 23 μ l of the master mix and 2 μ l undiluted cDNA were transferred to each of the tubes after which the tubes were capped.

The PCR tube was transferred into the real-time PCR machine. To activate the enzyme, the samples were incubated at 95°C for 15 minutes. The cycling was carried out at 95°C for 15 seconds with an annealing temperature of 50°C for 30 seconds. The cycles were repeated 45 times.

For primer and probe sequences, see Table 3.

Normalisation of qPCR data

The reference genes were used to analyze the IFN expression. First, the individual C_T-values (IFNs as well as reference genes) were inverted, by using the follow equation: C_{max}-C_T, where C_{max} is the total number of cycles for that particular run. Then, the mean values for the gene of interest were divided by the mean value of the three reference genes.

Results

RNA extraction from the brain tissue

The extraction of RNA from homogenised brain tissue resulted in a high yield of RNA that could be used for purity check (NanoDrop) and PCR measurements. The exchange of RNA from the homogenised tissues was efficient, and the purity measured at 260/280 by NanoDrop.

Reference genes

Six reference genes were used in the initial evaluation (Tables 2 & 3 in Material and Methods). However, only three of the reference genes were stable enough to be used for the normalisation of IFN expression, i.e., HPRT, RPS7 and YWHAZ. These reference genes were thus used for another duplicate run with the SYBR-green PCR. Results obtained from the reference genes were used for calculations of changes in gene expression of the cytokines. In the horse brain no gene expression of any cytokine could be detected, as expected, showing a high degree of species specificity for the IFN qPCR.

IFN-expression

All cats, 25 samples and one horse, were run in duplicate with two samples analysed from each brain for IFN- γ . These results are shown in comparison to the histopathological results (Table 1). IFN- α and - β expression were also analysed. However, since positive results were obtained in the -RT reaction, these analyses were only performed once.

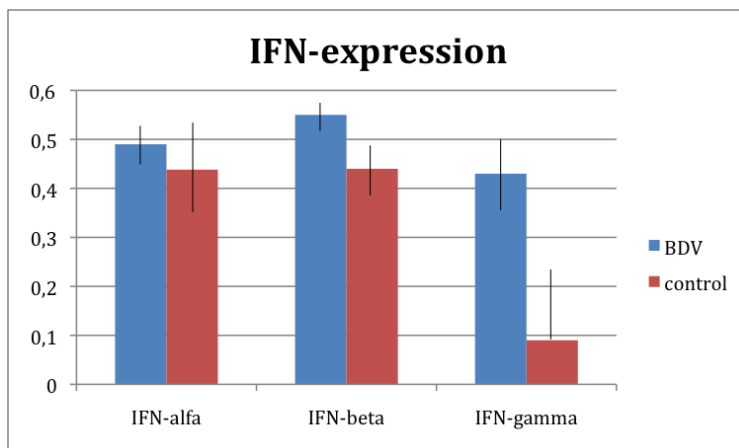


Figure 1. Normalised IFN- α , - β and - γ expression of the two group of cats. Mean values and standard deviations from duplicates run in at least one PCR-run are shown.

However, results of IFN- γ could be detected in the cat brain and clearly showed that cats with diagnosed Borna disease had a higher gene expression of IFN- γ in their brain compared to non virus diagnosed cats (Figure 1). According to Figure 1 cats with diagnosed Borna disease had a mean value of 0.43 ± 0.069 , comparing to the non-virus diagnosed cats that had a mean value of 0.09 ± 0.15 . IFN- α and - β showed similar results between the two groups of cats and it was not possible to obtain any valuable gene expression data because even the -RT samples showed positive responses even though they should have been negative (Figure 1). According to Figure 1 cats with diagnosed Borna disease the mean value for IFN- α and IFN- β were 0.49 ± 0.035 and 0.55 ± 0.037 , respectively. Similarly, the non-virus diagnosed cats had a mean value of 0.44 ± 0.069 , respectively.

The result of IFN- γ expression was verified with electrophoresis (Figures 2 and 3). The IFN expression was normalised using three reference genes (HPRT, RPL7 and YWHAZ). Normalised data are shown in Figure 1.

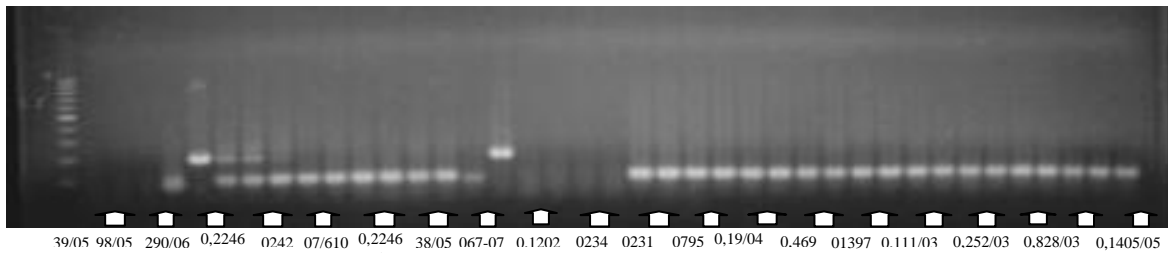


Figure2. Gel picture (first part) with IFN- γ duplicate mRNA analysed with TaqMan-PCR (100 kb). (More information about the samples can be seen in table 1)

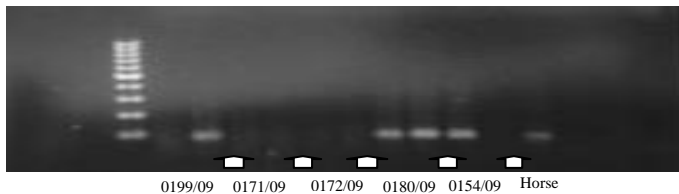


Figure3. Gel picture (second part) with IFN- γ mRNA analysed with TaqMan-PCR.

BDV-PCR

Real-time PCR was also used for detection of BDV. The histopathological finding showed that more than 13 cats suffered from staggering disease but regarding to the BDV-PCR only two cats were positive for BDV. However, all other cats analysed were regarded negative, as shown in Table 1.

Discussion

One major finding in the present study was that IFN- γ could be detected in the cat brain and clearly showed that cats with diagnosed Borna disease had a higher gene expression of IFN- γ in their brain compared to the control group. Another and notable finding was that the expression of IFN- α and IFN- β were similar between the two groups (Figure 1).

For these discoveries several different reference genes were used. The choice of reference genes was based on getting reliable results and highly accurate normalizations. Therefore HPRT, RPS7 and YWHAZ were picked, since they were more stable than the other reference genes and also encode different kinds of proteins. Hence, these genes will most probably be expressed independently of each other.

IFN- α and - β duplicates were run by PCR but it was not possible to obtain any valuable gene expression data since some of the -RT samples showed positive responses, even though they should have been negative. A reason for this could be contamination with DNA. Other possible errors are that one of the earlier extraction steps was not properly prepared or that the transformation from RNA to cDNA was not complete. Therefore no more tests were done with the IFN- α and - β .

In addition, it is known that IFN- α and - β are situated on one exon which means that it is very difficult to differentiate mRNA from DNA. However, this is not the case for IFN- γ and is one explanation why the method worked well for the study of IFN- γ expression.

Real-time PCR was also used for detection of BDV. Two cats were positive for BDV, however, all other cats were regarded negative (Table 1). One possible reason for these results was that gene-specific primers were not used during the cDNA synthesis, where oligo(dT)-primers were used. Therefore, only viral mRNA could be detected and it is likely that this highly affected the sensitivity. One other possible explanation could also be that during a virus infection, IFN- γ is released and that will reduce the viral replication in the brain. This hypothesis seems more likely since the necropsy indicated that more than two cats had typical signs of BDV-infection, i.e., non-purulent inflammation of CNS.

Real-time PCR results showed that Borna disease diagnosed cats had compared to non virus disease diagnosed cats a high gene expression of IFN- γ with similar C_T -values within the group of diseased cats. Moreover, gel electrophoresis data showed that the correct PCR-product for IFN- γ was obtained (Figures 2 and 3).

This IFN- γ PCR was performed twice to make sure that the results were comparable for each run and that no contamination or errors had been made during the analysis. When comparing the C_T -values of IFN- γ with the histopathological finding it was clearly evident that Borna disease diagnosed cats had a much higher gene expression than the other cats (Figure 1). However, a majority of non virus diagnosed cats showed C_T -values above 38. Consequently, results showed clear association between Borna disease diagnosis and increased IFN- γ gene expression. Since IFN- γ is known to be activated and important for the anti-viral response, it is reasonable to assume that Borna disease diagnosed cats had an ongoing active infection, including virus-induced inflammatory processes. It is also known that IFN- γ interacts with different kinds of cell types in the immune system and will therefore inhibit or stimulate proteins inside the cytokine network. This could lead to an increased viral infection or a slower processing of the infection in the brain (Consales *et al*, 1990). Even

though some of the cats were regarded as virus negative an IFN- γ expression could be shown in the brain, for example O.1405/05 M. Spinal, see Table 1. The reason may be that these cats showed inflammatory lesions which are associated with immune cell activation and production and release of cytokines.

In conclusion, it seems that IFN- γ in the brain is a reliable study of factors affecting the health and the immune system, in cats with feline Borna disease.

References:

- Afonina, I., Ankoudinova, Mills, A., Lokhov, S., Huyng, P., Mahoney, W., 2007, Primers with 5' flap improve real-time PCR, *BioTechniques* 43, 770-774.
- Abbas, A., Lichtman, A., 2003, *Cellular and molecular immunology*, Saunders, fifth edition.
- Berg, A-L., Reid-Smith, R., Larsson, M., Bonnett, B., 1998, Case control study of feline Borna disease in Sweden, *The veterinary record* 142,715-17.
- Berg, M., Johansson, M., Montell, H., Berg, A., 2001, Wild birds as a possible natural reservoir of Borna disease virus, *Epidemiology and Infection* 127, 173-178.
- Chan, E., Antonishyn, N., McDonald, R., Maksymiw, T., Pieroni, P., Nagle, E., Horsman, G., 2002, The use of TaqMan PCR Assay for detection of *Bordetella pertussis* Infection From Clinical Specimens, *Archives of Pathology and Laboratory Medicine* 126, 173-176.
- Consales, CA., Mendonca, RZ., Lucciari, MA., Vassao, RC., Pereira, CA., 1990, Macrophage activity in rabies virus infection of genetically selected high and low antibody responder lines of mice, *Research in Virology* 141, 57-67.
- Degiorgis, M., Berg, A., 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.
- Foley, J.E., Rand, C., Leutenegger, C., 2003, Inflammation and changes in cytokine levels in neurological feline infectious peritonitis, *Journal of Feline Medicine and Surgery* 5, 313-322.
- Hans, A., Bajramovic, J., Syan, S., Perret, E., Dunia, I., Brahic, M., Gonzalez-Dunia, D., 2004, Persistent, non-cytolytic infection of neurons by Borna disease virus interferes with ERK 1/2 signaling and abrogates BDNF-induced synaptogenesis, *The FASEB Journal express article* 10, 03-0764.
- Kamhieh, S., Flower, RL., 2006, Borna disease virus (BDV) infection in cats. A concise review based on current knowledge. *Veterinary quarterly* 28, 66-73.
- Kinnunen, P., Billich, C., Ek-Kommonen, C., Henttonen, H., Kallio, E., Niemimaa, J., Palva, A., Staeheli, P., Vaheri, A., Vapalahti, O., 2007, Serological evidence for Borna disease virus infection in humans, wild rodents and other vertebrates in Finland, *Journal of Clinical Virology* 38, 64-69.
- Leutenegger, C., Mislin, C., Sigrist, B., Ehrenguber, M., Hofmann-Lehmann, R., Lutz, H., 1999, Quantitative real-time PCR for the measurement of feline cytokine mRNA, *Veterinary Immunology and Immunopathology* 71, 291-305
- Livak, J, L., Schmittgen, D, T., 2001, Analysis of Relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method, *Methods* 25, 402-408.

Ludwig, H., Bode, L., 2000, Borna disease virus: new aspects on infection, disease, diagnosis and epidemiology. *Revue Scientifique et technique* 19, 259-88.

Penning, L., Vrieling, H., Brinkhof, B., Riemers, F., Rothuizen, J., Rutteman, G., Hazewinkel, H., 2007, A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues, *Veterinary immunology and immunopathology* 20, 212-222.

Pfaffl, M., 2001, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic acids research* 29, 45.

Staheli, P., Sauder, C., Hausmann, J., Ehrensperger, F., Schwemmle, M., 2000, Epidemiology of Borna disease virus. *Journal of general virology* 81, 2123-2135.

Wensman, J., Thorén, P., Hakhverdyan, M., Bélak, S., Berg, M., 2007, Development of a real-time RT-PCR assay for improved detection of Borna disease virus, *Journal of Virological Methods* 143, 1-10.