Novel Molecular Method for Detection of Bovine-Specific Central Nervous System Tissues as Bovine Spongiform Encephalopathy Risk Material in Meat and Meat Products

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The emergence of a new variant of Creutzfeldt-Jacob disease during the bovine spongiform encephalopathy epidemic has focused attention on the use of tissues from the central nervous system (CNS) in food. For efficient consumer protection, European legislation prohibits several bovine tissues, encompassing mainly the central nervous system, from the food chain. A quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was designed to identify bovine spongiform encephalopathy risk material in meat and meat products. This was based on an mRNA assay that used bovine, ovine, and caprine glial fibrillary acidic protein (GFAP) encoding gene sequences as a marker. The real-time RT-PCR assay allowed the detection of bovine, ovine, or caprine CNS tissues in meat and meat products. Bovine brain at a concentration of 0.01% yielded a positive PCR reaction. The real-time RT-PCR assay included a housekeeping gene as an endogenous control. The detection was not affected by heat treatment of the meat products. The quantitative real-time RT-PCR detection of GFAP mRNA appeared to be useful as a routine diagnostic test for the detection of illegal use of CNS tissues in meat and meat products. The stability of the specific region of GFAP mRNA also allows the detection of CNS tissues after meat processing steps. The use of organ- and species-specific subunits of mRNA might be a promising approach for the detection of other banned tissues. (*J Mol Diagn* 2005, 7:368–374)

Since 1996, evidence has been increasing for a causal relationship between ongoing outbreaks in Europe of a bovine spongiform encephalopathy (BSE) in cattle and the variant Creutzfeldt-Jakob disease in humans. However, some member states are not able to give an exact overview about the occurrence and the development of BSE disease. This is based in part on the late start with

area-wide BSE tests and on the delayed implementation of prohibition of meat and bone meal for animal feeding. In the U.S., the first BSE case was announced in December 2003.¹ However, there is strong evidence that BSE can cause variant Creutzfeldt-Jakob disease, most likely via the oral route of infection.^{2–4} Public health concerns require the efficient exclusion of ruminant tissues containing high accumulations of the causative agent (PrP^{Sc}),⁵ particularly brain and spinal cord, from the food chain. This is demanded by European laws. It should be mentioned that porcine central nervous system (CNS) is not affected by this legislation.⁶

For further risk assessment, the member states are classified according to their individual situation of reported and expected BSE cases. Hence, for the maintenance of the “BSE-free status” of the affected countries and the strong prohibition of specified bovine offal from the food chain, the detection of BSE risk material is one of the highest priority tasks for food analysts. Several phenotypic methods for detection of BSE risk material have been developed, including enzyme-linked immunosorbent assay, HPLC, Western blot, and immunohistochemical methods.^{7–11} However, these methods allowed neither differentiation of CNS tissues of banned species from CNS tissues of other animal species nor an exact quantification of the detected CNS. In addition, a test system should also allow an effective control of heat-treated samples.

In the present study, a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method based on a species-specific glial fibrillary acidic protein (GFAP) mRNA region was developed for the detection of bovine, ovine, and caprine CNS tissues in raw and heat-treated meat products. The relative quantitative technique was evaluated in CNS and other tissues of various animal origins.

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Materials and Methods

Samples and the Internal Reference Material (IRM)

The raw meat samples used in the present investigation were purchased from a slaughterhouse in Giessen, Germany. The materials used were muscle ($n = 3$), liver ($n = 3$), heart ($n = 3$), kidney ($n = 3$), lung ($n = 3$), spleen ($n = 3$), lymph nodes ($n = 3$), peripheral nerves (in particular sciatic and axillaries nerves) ($n = 6$), and spinal cord ($n = 3$) from bovine subjects as well as muscle of ovine, caprine, and porcine origin. In addition, brains from bovine ($n = 10$), ovine ($n = 3$), caprine ($n = 2$), and porcine ($n = 5$) subjects were included. These were removed directly from skulls; porcine brains were taken from carcasses of pig halves. The samples were transported immediately on ice to the laboratory. Additionally, brain tissues from chicken ($n = 2$), turkeys ($n = 2$), and ducks ($n = 1$) were kindly provided by the Klinik für Vögel, Reptilien, Amphibien und Fische, Justus-Liebig-Universität Giessen. Likewise, cooked and raw sausages with varying additives of bovine brain ($n = 4$ of each) were produced as IRM according to the basic recipes provided by the guiding principles of the German Food Code.¹² The preparation of the sausages is described by Lückner et al.⁹ The sausage filling contained 50% porcine meat (max 10% fat), 5% bovine meat, 25% porcine fat, 20% ice, 20 g/kg curing salt (4 to 5 g/kg NaNO₂ in NaCl; Enders, Reiskirchen, Germany), 3 g/kg phosphate-based stabilizer (di-Na-phosphate; Kirchheimbolanden, Germany), 5 g/kg ready mixed spices for cooked sausages (Delikatess Aufschnitt, Gewürzmüller, Stuttgart, Germany), and bovine brain in varying concentrations (20, 8, 1, 0.1, and 0.01%). For preparing the cooked sausages, the contents were heated for 90 minutes at 80°C in a steam heater (Fessmann, Winnenden, Germany).

DNA Extraction

Total cellular DNA was isolated from bovine, ovine, caprine, and porcine muscle samples using the DNeasy tissue isolation kit (Qiagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 25 mg of the sample was lysed, followed by binding of the DNA to the spin column (Qiagen). After washing steps, the DNA was eluted with 100 μ l of elution buffer; 2.5 μ l was used as a DNA template.

Amplification of GFAP-DNA Subunits

A segment of bovine, ovine, caprine, and porcine GFAP gene was amplified and sequenced. For this purpose, sequencing primers were designed using human (accession number AF028784), rat (accession number Z48978), and bovine (accession number Y08255) GFAP gene sequences already published in National Center for Biotechnology Information GenBank.

The PCR reaction mixture (50 μ l) contained 1 μ l of primer 1 (10 pmol/ μ l), 1 μ l of primer 2 (10 pmol/ μ l), 1 μ l

of dNTP (10 mmol; Roche Diagnostic, Mannheim, Germany), 5 μ l of 10 $\times$ thermophilic-buffer (Applied Biosystems, Darmstadt, Germany), 0.2 μ l of TaqDNA polymerase (5 U/ μ l; Applied Biosystems), and 39.3 μ l of double-distilled water. Finally, a 2.5- μ l of DNA preparation was added to each reaction tube. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600; Applied Biosystems) with the following program: one 3-minute precycle at 93°C; and 35 times for 30 seconds at 93°C, 30 seconds at 52°C, and 45 seconds at 72°C, followed by a final extension incubation of 72°C for 5 minutes. The presence of PCR products was determined by electrophoresis of 10 μ l of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany) with Tris acetate-electrophoresis buffer (0.04 mol/L Tris and 0.001 mol/L EDTA, pH 7.8) and a 100-bp DNA ladder (Roche) as molecular marker.

Sequencing of GFAP Subunit

Sequencing of the amplified subunits of bovine, ovine, caprine, and porcine GFAP gene was performed using the facilities of the university (Institut für Medizinische Mikrobiologie und Virologie, Justus-Liebig-Universität Giessen) with the MegaBACE 1000 DNA Sequencing System (Amersham Pharmacia Biotech, Freiburg, Germany) with protocols described by the manufacturer. The sequence data were further studied and analyzed with the computer program SeqMan (Lasergene; DNASTAR, Inc., Madison, WI).

Design of Specific Oligonucleotide Primers and the Fluorogenic Probe

Based on the generated bovine, ovine, caprine, and porcine GFAP gene sequences, a GFAP gene region was determined to be characteristic for the three ruminant species but not for porcine GFAP gene. The specific oligonucleotide primers were designed using the Primer Express Software (version 2.0; Applied Biosystems). The forward primer RTGcowM56F2a was selected from a region located on exon 5, whereas the reverse primer RTGcowM56R2a was selected from a region of exon 6. To avoid the amplification of DNA, the TaqMan fluorogenic minor groove binder (MGB)-probe was selected in the exon 5/exon 6 junction region and conjugated with 6-carboxy-fluorescein (FAM). The sequences of the oligonucleotide used in the real-time PCR reaction were as follows: the forward primer RTGcowM56F2a, 5'-ACC TGC GAC CTG GAG TCC T-3'; the reverse primer RTGcowM56R2a, 5'-CTC GCG CAT CTG CCG-3'; and the fluorogenic MGB probe OptiR, 6-FAM-ACT CGT TCG TGC CGC GC-MGB. The oligonucleotide primers and the fluorogenic probe were synthesized by Applied Biosystems.

RNA Extraction

Total cellular RNA was isolated from the samples using the RNeasy Lipid Tissue mini kit (Qiagen) according to

the manufacturer's instructions. Briefly, 1000 μ l of Qiazol was added to a 50-mg sample, and the mixture was transferred to a glass matrix tube (FastRNA Green; Q BIOgene, Heidelberg, Germany) for cell lysis. The mixture was processed in a spin/rotation instrument for cell lysis (FastPrep-120; Q BIOgene), with a speed setting of 6 and a time setting of 45 seconds. After processing, 200 μ l of chloroform was added to the mixture. The aqueous and organic layers were separated by microcentrifugation for 15 minutes at room temperature at $10,000 \times g$. The aqueous phase, containing the RNA, was removed and 200 μ l of ethanol (70%) was added, followed by binding of the RNA to the spin-column (Qiaagen). After DNase digestion with 80 μ l of RNase-free DNase (Qiaagen), the total RNA was washed and eluted with 50 μ l of elution buffer.

Real-Time RT-PCR Analysis

Total RNA from each sample was subjected to reverse transcription using TaqMan Reverse Transcriptase Reagents kit with uracil-*N*-glycosylase (Applied Biosystems) according to the manufacturer's protocol. The reactions were incubated at 25°C for 10 minutes and 48°C for 30 minutes followed by a final reverse transcriptase inactivation at 95°C for 5 minutes. Real-time PCR reactions were subsequently carried out in a 50- μ l reaction mixture with final concentrations of 300 nmol/L of each oligonucleotide primer, 200 nmol/L of the fluorogenic probe, and 1 $\times$ TaqMan Universal PCR Master mix (Applied Biosystems) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Thermal cycling conditions comprised an initial UNG incubation at 50°C for 2 minutes, an AmpliTaq Gold DNA Polymerase activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and an annealing and extension at 60°C for 1 minute. Each measurement was performed at least in duplicate, and the threshold cycle (C_t) (the fractional cycle number at which the amount of amplified target reached a fixed threshold) was determined.

Construction of Standard Curve

The GFAP cDNA concentration was estimated by using an UV spectrophotometer (DU 640; Beckman). Real-time PCR amplifications of the serially diluted GFAP cDNA were performed using the same PCR conditions mentioned above. The C_t value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). All of the reactions were run in triplicate, and the normalized reporter signal ΔR_n and C_t were averaged from the values obtained in each reaction. A standard curve was then constructed by plotting the C_t of known concentration of each standard sample. The quality of the standard curve can be judged from the slope and the correlation coefficient (r). The slope of the line can be used to determine the efficiency of the target amplification (Ex) using the equation $Ex = (10^{-1/\text{slope}}) - 1$.¹³

Endogenous Control and Relative Quantitative Analysis

The comparative C_t method was used for the relative quantitative detection of the expression of GFAP gene in different organs with the 18S rRNA as an endogenous control for each reaction. The 18S rRNA probe was labeled with the fluorescent dye FAM (Assay on Demand; Applied Biosystems). Real-time data were analyzed using the comparative C_t method. This method is similar to the standard curve method, except that it uses the arithmetic formula $2^{\Delta\Delta C_t}$ to achieve relative quantification (Applied Biosystems User Bulletin 2). The relative expression of GFAP gene in the different organs was determined with reference to the muscle after normalization against 18S rRNA as implemented in the ABI PRISM 7000 Sequence Detection System software.¹⁴ A prior validation experiment was performed to demonstrate that amplification efficiencies of GFAP cDNA and 18S rRNA primer/probe sets.

Results

Sequences of GFAP Gene Subunit

According to the published human, rat, and bovine GFAP gene sequences, oligonucleotide primers were designed that allowed an amplification of subunits of bovine, ovine, caprine, and porcine GFAP genes. These partial sequences were used for the design of specific oligonucleotide primers and a fluorogenic probe and were published in GenBank under the accession numbers AY617158, AY617159, AY617160, and AY617161, respectively.

Specificity of the Real-Time GFAP RT-PCR

The designed oligonucleotide primers RTGcowM56F2a and RTGcowM56R2a allowed after reverse transcription an amplification of specific regions of bovine, ovine, and caprine GFAP mRNA isolated from brain with a size of 86 bp. No amplification could be observed with GFAP mRNA of porcine origin. Amplification of a bovine DNA preparation as positive PCR control yielded an amplicon with a size of 219 bp (Figure 1). The oligonucleotide primers were subsequently used together with a fluorogenic MGB probe for TaqMan RT-PCR detection of GFAP mRNA. The real-time RT-PCR amplified bovine, ovine, and caprine GFAP mRNA, but not bovine DNA or porcine mRNA and DNA. In addition, mRNA or DNA from turkey, chicken, and duck brain tissues showed no positive signal.

Evaluation of Housekeeping Gene as an Endogenous Control and Determination of the Detection Limit of the Real-Time GFAP RT-PCR

Using a serial dilution of a known quantity of a bovine brain GFAP cDNA standard, ranging from 9.66E – 04 to

Figure 1. Typical amplification products of bovine (lane 1), ovine (lane 2), and caprine (lane 3) GFAP cDNA with a size of 86 bp; negative RT-PCR of porcine GFAP cDNA (lane 4); positive PCR control by using genomic DNA with a size of 219 bp (lane 5); and negative control (lane 6); M, DNA molecular weight marker XIII 50-bp ladder (Roche).

96.67 ng/PCR, it was possible to obtain a series of amplification plots and to determine their relative C_t values. Standard curves were then constructed by plotting the C_t of the known concentration of each standard sample with the GFAP and 18S rRNA RT-PCR. Each sample was performed in triplicate, and the standard curves showed slopes of -3.561 and -3.630 , respectively. The correlation coefficient (r) was >0.999 of both GFAP RT-PCR and 18S rRNA RT-PCR. This indicated that relative differences in target genes could be calculated by the comparative C_t method (Figure 2). Because the C_t value decreased linearly with the increasing amount of GFAP cDNA concentration in the standard sample, the absolute quantity of the GFAP transcript in an unknown sample could be accomplished by measuring the C_t value and using the standard curve to calculate the absolute GFAP cDNA concentration. The detection limit of this real-time GFAP RT-PCR system was approximately 1.0×10^{-12} g/PCR reaction of absolute GFAP cDNA. This concentration could be detected by a C_t value of around 36 cycles (Figure 3).

Figure 2. Relative standard curves show comparable amplification efficiencies of GFAP gene (target) PCR and 18S rRNA gene (housekeeping gene) real-time RT-PCR. The Δ slope was <0.1 .

Figure 3. Real-time GFAP RT-PCR amplification curves of undiluted and 10-, 100-, 1000-, 10,000-, and 100,000-fold dilutions of bovine cDNA. On the y axis, the absolute emission intensity is indicated; the x axis shows the number of PCR cycles. The calculated cDNA concentrations are shown in the table.

Organ Specificity and the Relative Quantification of Real-Time GFAP RT-PCR

The mRNA of GFAP gene of three different samples of brain, spinal cord, peripheral nerves, liver, lung, spleen, heart, lymph node, and muscle from bovine subjects was determined in triplicate transcripts by relative quantitative real-time PCR. For all samples, the expression level of the GFAP gene was calculated relative to the organ muscle in the presence of the endogenous control (18S rRNA gene). The endogenous control shows a positive signal with all cDNA samples investigated. The C_t values of all non-neural organs investigated were greater than 35 cycles. The values of the relative amount of GFAP cDNA obtained from non-neural organs varied between $2.00E - 03$ with kidney samples to $3.90E + 00$ in investigating lymph node samples. The CNS tissues including brain and spinal cord contained $1.46E + 05$ and $1.88E + 06$, respectively. The relative amount of peripheral nerves was $2.41E + 02$ (Table 1). No fluorescent signal was detected in any of DNA samples of the various organs or in the negative RT-PCR control. Figure 4 demonstrates the $\log_{10}$ values of the relative amount of the GFAP expression in different organs investigated.

Detection of Bovine GFAP in IRM

To test the stability and detectability of bovine GFAP mRNA after meat processing and heat treatment, cooked and raw sausages with varying additives of bovine brain (20, 8, 1, 0.1, and 0.01%) were investigated by quantitative real-time PCR (Figure 5). The bovine brain was detectable in raw as well as in heat-treated sausages. The C_t values of the raw sausages with various brain concentrations ranged from 20.17 cycles for sausages containing 20% bovine brain to 32.23 cycles for sausages that comprised 0.01% of bovine brain. The C_t values of the cooked sausages ranged from 20.18 cycles for sausages containing 20% bovine brain to 33.42 cycles for sausages with 0.01% of bovine brain. The bovine DNA and the negative control as well as the sausages without

Table 1. Relative Quantitative Detection of GFAP mRNA in Different Bovine Organs Using Comparative C_t Method ($\Delta\Delta C_t$)

Organs	GFAP	18S rRNA	GFAP	$\Delta\Delta C_t^{\dagger}$	GFAP	
	Average C_t	Average C_t	Average ΔC_t^*		RQ to Muscle [‡]	RQ $\log_{10}^{\S}$
Brain	18.445	14.349	4.096	-17.154	1.46E + 05	5.16
Spinal cord	14.737	14.327	0.410	-20.840	1.88E + 06	6.27
Peripheral nerves	29.206	15.867	13.339	-7.911	2.41E + 02	2.38
Heart	35.813	14.582	21.231	-0.019	1.01E + 00	0.01
Kidney	46.851	16.697	30.154	8.904	2.00E - 03	-2.68
Liver	46.042	15.077	30.965	9.715	1.00E - 03	-2.92
Lung	36.514	14.703	21.811	0.561	6.78E - 01	-0.17
Lymph node	36.105	16.819	19.286	-1.964	3.90E + 00	0.59
Muscle	36.234	14.984	21.250	0.000	1.00E + 00	0.00
Spleen	37.669	14.663	23.006	1.756	2.96E - 01	-0.53

*GFAP ΔC_t is the C_t for the target gene normalized to an endogenous control (18S rRNA) ($C_{t\text{ GFAP}} - C_{t\text{ 18S rRNA}}$).

[‡]The calculation of $\Delta\Delta C_t$ involves subtraction by the ΔC_t calibrator value (muscle) ($\Delta C_{t\text{ organ}} - \Delta C_{t\text{ muscle}}$).

[†]Relative quantitative of GFAP determined by this expression: ($2^{-\Delta\Delta C_t}$).

[§]Amount of the target GFAP relative to the calibrator as $\log_{10}$ of the $\Delta\Delta C_t$ value.

added bovine brain yielded a fluorogenic signal under the threshold level (Table 2). The endogenous control shows a positive signal with all cDNA of IRM investigated even after heat treatment.

Discussion

In the present study, a sensitive detection assay for bovine, ovine, and caprine CNS tissues was developed using an mRNA-based quantitative real-time RT-PCR with the GFAP gene as marker. This protein is expressed at high levels in the astrocyte cells of the CNS tissues but not or only at very low levels in the cellular elements of other organs and peripheral nerves.⁷ GFAP protein had been previously used as a marker for the detection of CNS tissues. A detection was performed by Western blotting,^{9,10} by enzyme-linked immunosorbent assay,^{7,8} and by immunohistochemistry.¹¹ The disadvantage of these GFAP detection methods is that none of these methods appeared to be species specific. A species specificity could be determined by gas chromatography spectrometric analysis of the fatty acid composition of meat components¹⁵ or by PCR-RFLP analysis of GFAP mRNA.¹⁶ However, the spectrometric analysis requires expensive laboratory equipment, and the latter shows cross reactions with other organs, eg, muscle or heart. In addition, the PCR-RFLP method used a conventional detection by nonquantitative gel electrophoresis.

Figure 4. Relative quantitative value ($\log_{10}$) of different organs calculated relative to the organ muscle, normalized to the housekeeping gene.

In the present study, partial gene sequences of bovine, ovine, caprine, and porcine GFAP genes were analyzed to find conserved and variable regions. These regions were used for the selection of species-specific oligonucleotide primers and a fluorogenic probe to amplify RT-PCR products that allow an amplification of species-specific regions of mRNA of bovine, ovine, and caprine origin but not of mRNA of porcine origin. The assay allowed the detection of CNS tissues and in parallel the identification of the species. An mRNA-based analytical test to determine the presence of CNS tissues in meat should be sensitive and should reliably avoid false-positive results of DNA contamination. Based on this consideration, we designed a MGB TaqMan probe, which was selected in the junction of exon-exon region. The presented real-time RT-PCR amplified and detected only mRNA but not bovine or porcine DNA.

The sample preparation is one of the most critical aspects of mRNA assays because this might cause false-negative results. The RNA isolation method, using a combination of a mechanical step (Fast Prep) and the RNeasy Lipid Tissue kit, as performed in this study provided a good quality of total RNA. The high lysis efficiency of this method enables extraction of a sufficient amount of total RNA in about 1 hour. In comparison with classical RNA extraction protocols, it has been shown that the selected method increased the sensitivity of the detection of GFAP mRNA in meat products (data not shown).

The typical standard curves for the quantitative real-time must be generated with an optimal correlation coefficient of >0.99 . The efficiency of cDNA synthesis from an mRNA template is important for the downstream processing of cDNA quantitation. The relative quantitation standard was reverse transcribed in a batch process that included the RNA samples to be quantitated. This procedure allowed for accurate quantitation between different assays, regardless of differences in reverse transcription efficiency, as aliquots of the same standard RNA were used. The endogenous control for quantitative RT-PCR experiments functions as a control for reverse transcription and PCR reaction, as well as mRNA quantity, quality, and integrity. rRNAs are frequently used as internal controls for quantification experiments, particularly

Figure 5. Detection of bovine GFAP mRNA in sausages with varying quantities of homogenized brain tissue added with (→) and without (→) heat treatment of the samples.

because stable expression levels of 18S rRNA relative to other housekeeping genes have been described for rat, mouse, and human tissues.^{17,18} However, 18S and 28S rRNA are distinct from messenger RNAs, constituting up to 80% of total cellular RNA.¹⁹ The correlation coefficients for standard curves and efficiency values for detection of GFAP and 18S rRNA gene were excellent. The mean correlation coefficient for these response curves obtained was >0.999 . The efficiency of amplification was very similar (efficiencies were within 0.1). According to the results of this study the 18S rRNA gene can be recommended to serve as an endogenous control to avoid the false-negative results as well as for relative quantitative detection of GFAP gene in different tissues. The detection limit of this system was $<0.01\%$ of CNS tissues in meat products, which is 100 times less than the detection limit of two previously described commercial kits.²⁰ In addition, both of these detection kits are based on immunological reactions and are not species specific.

In general, mRNA can have a short half-life within viable cells and is rapidly degraded by specific enzymes (RNases), which are themselves very stable even in environments outside the cell itself.²¹ It has been previously reported, that mRNA sequences from *Escherichia coli* could be amplified for up to 30 hours after cell death.²² In mammalian cells, the abundance of a particular mRNA can be many folds more than in bacteria. It has been demonstrated that mRNA degradation can be dependent on the sequence content²³ or regions thereof.²¹ In the present study, the functional activity or the structural integrity of the GFAP mRNA was not investigated; only a small species-specific region of GFAP mRNA has been used as a marker of CNS tissues. This can explain the stability of this region during the food processing steps even after heat treatment.

For determination of expression level of GFAP mRNA in different organs, $2^{\Delta\Delta C_t}$ method was used, which is included an endogenous control and a calibrator organ. The purpose of the endogenous control is to normalize the PCRs for the amount of the mRNA added to the reverse transcription reactions. The choice of calibrator for $2^{\Delta\Delta C_t}$ method depends on the type of target gene. The data are presented as the fold change in gene expression normalized to the 18S rRNA gene and relative to the calibrator organ (in our case muscle as a primary component of the meat product). The present assay detected very low levels of GFAP in non-neural organs. The relative amount of GFAP expression obtained from all of these organs was less than $3.90E + 00$. The peripheral nerve samples yielded a relative value of $2.41E + 02$. This was in contrast to brain and spinal cord values of $1.46E + 05$ and $1.88E + 06$, respectively; corresponding to about 1000- and 10,000-fold more of GFAP mRNA content. The results of the GFAP levels in the different neural tissues obtained in the present study were comparable with the results demonstrated previously by other authors.^{7,8}

The C_t value obtained from all of these organs was above 35 cycles, whereas a concentration of 0.01% of brain exhibited a signal at C_t value of 32.23 (± 0.12) cycles. The peripheral nerve samples yielded a C_t value of 29.21 (± 0.10). The C_t values of brain and spinal cord were 18.45 (± 0.01) and 14.74 (± 0.16), respectively.

The peripheral nerve signal does not present a realistic problem in the analysis of CNS detection in meat and meat products. Peripheral nerves would be detected if present as one of the primary components of the meat product. Thus, if the peripheral nerves comprised more than 10% of the meat product, a fluorescent signal would be detected at the C_t value of 34.00 (± 1.20) cycles; this is equivalent to 0.047 ng cDNA/PCR reaction (data not shown). This signal is about one-seventeenth of the concentration value of 3.28 ng cDNA/PCR resulting from 0.1% brain. Therefore, the use of a standard curve and a cut-off value of significant CNS contamination of about 0.1% will circumvent this problem.

Analysis of IRM containing bovine brain homogenate and of bovine brain homogenate alone revealed GFAP mRNA RT-PCR signal stability. Enzymes released from minced muscle and brain tissue apparently did not influence the detectability of the bovine GFAP mRNA signal. Analogous results were found for the study of a heated meat product with varying amounts of bovine brain homogenate added. Ingredients and additives commonly used in sausages (see Materials and Method) and other meat products also did not influence the detection of

Table 2. Detection of Bovine GFAP mRNA in Sausages with Varying Quantities of Homogenized Brain Tissue with and without Heat Treatment of the Samples

IRM	C_t values of investigated sausages with varying brain homogenate concentration					
	20%	8%	1.0%	0.1%	0.01%	0%
Raw	20.17	23.33	28.58	29.99	32.23	Undetected
Heat treated [†]	20.18	23.45	28.74	30.09	33.42	Undetected

* C_t value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold indicating the amplification of the target sequence.

[†]Heat treatment at 80°C for 90 minutes.

bovine CNS tissue. In addition, it was demonstrated that the applied pasteurization conditions (core temperature, 80°C) had no significant negative effect on the bovine GFAP mRNA signal. Because 0.01% bovine brain homogenate was successfully detected in all of the experiments conducted and no false-negative results were obtained, we conclude that the detection limit is below 0.01% and must be determined in a future study along with the influence of extended heating (eg, sterilization, 121°C) and a long storage period.

According to the present results, an efficient method was used to detect the presence of CNS tissues in meat mixtures containing a minimum amount of bovine CNS tissues. The small mRNA region of the bovine GFAP was not considerably affected by a prolonged storage and heat denaturation of the rendering process, and its sensitivity proved to be high also when samples were further subjected to 80°C for 90 minutes. On the basis of these results and previously published studies,^{7–11,16} it can be stated that bovine GFAP mRNA exhibits storage and heat stability.

In conclusion, quantitative real-time RT-PCR detection of GFAP appears to be useful as a routine diagnostic test for detection of the illegal use of bovine CNS tissue in meat and meat products. Bovine GFAP mRNA exhibits certain stability, facilitating the detection of CNS tissue in raw and heat-treated sausages. The technique should be evaluated through a ring trial to confirm the ability of the test in different laboratories. According to our knowledge, this is the first report of a real-time RT-PCR method for the species- and tissue-specific detection of bovine CNS tissue in meat and meat products. Furthermore, this approach seems to be promising for the species-specific detection of other banned tissue.

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