Research Article

High sensitivity detection of the glial fibrillary acidic protein as indicator for TSE risk material in meat products using an immuno-PCR

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The emergence of prion diseases in cattle during the bovine spongiform encephalopathy (BSE) epidemic and the transmission to humans causing variant Creutzfeldt – Jakob disease by consume of BSE-contaminated meat has focused attention on the use of tissues from the central nervous system (CNS) in food. To avoid food contamination, it is regulated by law that specified risk material has to be removed from food chains. Detection of well-expressed CNS indicator proteins such as the glial fibrillary acidic protein (GFAP) assumes an important role in detection of food contamination; however, the sensitivity of detection performed on basis of ELISAs is limited. Consequently, there is an urgent need for high-sensitivity detection methods. We describe the development of an immuno-PCR assay with enhancement of the immunoreaction by amplification of a DNA fragment linked to the antigen – antibody complex. This immuno-PCR assay shows enhanced sensitivity in GFAP detection of at least two orders of magnitude compared to the ELISA technique carried out under identical conditions. The immuno-PCR proves to be a suitable tool for the high-sensitivity detection of marker proteins in food imported by food chain processes. It is applicable for use by food and veterinary facilities, and institutes involved in food monitoring.

Keywords: Food contamination / GFAP / Immuno-PCR / Prion diseases

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1 Introduction

Transmissible spongiform encephalopathies (TSEs) are prion diseases affecting both humans and animals, *e.g.* the Creutzfeldt – Jakob disease (CJD) is the disease of humans, scrapie of sheep and goats and the chronic wasting disease of cervids. The bovine spongiform encephalopathy (BSE) of cattle has attracted worldwide attention due to the epidemic outbreak in the UK and the worldwide spread. Prion diseases are counted among food borne infections as proven by the transmission of BSE to cattle by feeding TSE infected meat and bone meal. Many lines of evidence link the transmission of BSE to humans causing variant CJD (vCJD) by consume of BSE-contaminated food and meat products [1 – 2]. The most efficient measure for preventing contamination of food chains with PrP^{Sc} is the removal of specified risk materials (SRM) that is demanded by European laws, especially in the case of tissues from the central nervous system (CNS) which contain the highest levels of infectivity.

The deposition of infectious prion protein aggregates (PrP^{Sc}) is the hallmark of this degenerative disease. PrP^{Sc} plays a major role in the transmission and development of the disease and there is substantial evidence that the protein is the major if not only agent causing TSE [3]. PrP^{Sc} compose of high β-sheet content and is specifically characterised by detergent insolubility. Its propagation results from a post-translational conversion into a conformational changed isoform of the naturally expressed cellular glycoprotein (PrP^{C}) in all tissues and cells. Using protease digestion, the two isoforms can be differentiated. Proteinase K (PK), a serine protease, completely hydrolyses PrP^{C} whereas PrP^{Sc} is fragmented at the amino-terminal region forming a resistant carboxyl-terminal core protein with a molecular mass of 27 – 30 kDa. Thus, remaining PrP^{Sc} proteins are a reliable marker for diagnosis the TSE, and sensi-

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Abbreviations: BSE, bovine spongiform encephalopathy; **GFAP,** glial fibrillary acidic protein; **SRM,** specified risk material; **TSE,** transmissible spongiform encephalopathy

tive techniques have been developed in recent years [4 – 8]. The preconditions for sensitive detection using these systems are, firstly, an adequate PrP^{Sc} accumulation in the tissue being analysed and, secondly, a high PK resistance. However, protease stability varies among prion strains and field isolates complicating doubtless diagnosis [9]. Of particular interest is the recent identification of PK-sensitive infectious PrP^{Sc} [10].

Food controls are in need for a high-sensitivity detection method for central nervous system (CNS) tissues in meat products. Several methods for SRM detection have been developed including Western blotting, ELISAs, GC-MS and RT-PCR detecting CNS markers as neurone-specific enolase (NSE), glial fibrillary acid protein (GFAP), myelin proteolipid protein (PLP) and fatty acids [11 – 19].

However, the immunological detection of CNS indicators is limited by factors affecting the antigen presentation, including the inadequate formation of antibodies and barriers affecting antibody accessibility to epitopes. Depending on antigenicity and antibody affinity, the protein detection using ELISA systems is limited to concentrations up to 10 pg/ml [20]. The sensitivity of the ELISA technique can be considerably enhanced using an immuno-PCR assay in which antibody binding is combined with DNA amplification. The immuno-PCR technique has been used to detect low-level proteins as bacterial and viral antigens as well as prions [21 – 29]. In this study, we describe our investigation on the development and evaluation of an immuno-PCR assay suitable for the ultrasensitive detection of GFAP as CNS indicator protein in meat products. Our assay achieved a sensitivity enhancement of at least two orders of magnitude when compared to ELISA carried out under identical conditions.

2 Materials and methods

2.1 Food and brain tissue suspensions

Beef and sausage as such or processed into meat products such as ground meat, corned beef, liver sausage, smoked meat, salami, tongue and goulash were acquired from local butcheries. Brain tissues were obtained from BSE-free cattle. Meat and brain tissues were weighed, homogenised in nine volumes Tris buffered saline (TBS, pH 7.4; Sigma, Taufkirchen, Germany) using pestles in glass tubes and homogenisers driven by electric motors (VWR, Darmstadt, Germany) followed by intensive ultrasonification for 30 – 60 s. Homogenate suspensions of 10% w/v were cleared by centrifugation at $1000 \times g$ for 5 min and supernatants were stored at $-20^{\circ}C$ until used.

2.2 Synthesis of biotinylated reporter DNA

A biotinylated 296 bp DNA fragment originating from plasmidal DNA of pUC19 (Fermentas, St. Leon-Rot, Germany) was synthesised by PCR using primers pUC-bio (5′-Biotin-CCC GGA TCC CAG CAA TAA ACC AGC CAG CC-3′) and pUC-2 (5′- GCC AAC TTA CTT CTG ACA AC – 3′) as described [23]. DNA was designed with insertion of a site for enzymatic restriction. A *Bam*HI site was included into the first primer.

2.3 Immuno-PCR assay

Protein suspensions were diluted in TBS prior to coating with 50 µl/well onto 96 well microtiter plates (Nunc Maxisorb, Roskilde, Denmark) for 16 h at $4^{\circ}C$. Antibody, streptavidin and DNA solutions were added to each well at a volume of 50 µl. Washing was carried out with volumes of 200 µl. Excess proteins were removed by washing five times with TBS containing 0.05% Tween 20 (TBST) followed by 30 min incubation in TBST and the washing procedure repeated at room temperature. For specific GFAP detection an appropriate dilution of the polyclonal antibody anti-GFAP (1:500; Dianova, Hamburg, Germany) was added to each well. This specific anti-GFAP antibody recognises the antigens of different species as cattle, sheep, humans and others. The antibodies were allowed to react for 2 – 3 h. Unbound antibodies were aspirated and wells were washed ten times with TBST. Biotin conjugated affinity purified goat anti-rabbit IgG (Dianova, Hamburg, Germany) was added as secondary antibody (final concentration 40 ng/ml) and allowed to react for 2 h at room temperature. Contents of the plates were aspirated and wells were washed ten times with TBST prior to the application of recombinant streptavidin (Roche, Mannheim, Germany) in a final concentration of 0.1 µg/ml. After 30 min incubation at room temperature, excess streptavidin was removed by washing ten times with TBST. Biotin-conjugated reporter DNA (5 pg/ml) was added to each well in order to bind to free sites of streptavidin for 30 min at $37^{\circ}C$. For blocking, unspecific DNA binding herring fish sperm DNA was added at a concentration of 1 mg/ml. Unbound DNA was removed by intensive washing with TBST (ten times) and TBS (five times). Bound DNA was released from the complex by enzymatic restriction using 1 U *Bam*HI in 50 µl restriction buffer (2 h, $37^{\circ}C$) and collected in new tubes. Aliquots (3 – 5 µl) were used as a template in conventional PCR. The PCRs were performed in a volume of 30 µl using a hot-bonnet thermocycler (Biometra, Göttingen, Germany). The mixture contained 200 µM of each deoxynucleosidetriphosphate, 15 pmol of primers pUC-1 (5′-CAG CAA TAA ACC AGC CAG CC-3′) and pUC-2, 3 µl of 10 fold concentrated polymerase synthesis buffer Y, 1.5 mM $MgCl_2$, and 0.65 U of Taq DNA polymerase (Peqlab, Erlangen, Germany). After 5 min initial denaturation at $95^{\circ}C$, PCR runs consisted of 26 cycles of denaturation ($95^{\circ}C$, 1 min), annealing ($55^{\circ}C$, 1 min) and extension ($72^{\circ}C$, 1 min), followed by final extension ($72^{\circ}C$, 5 min). In addition, a negative control containing no template DNA was included in each run.

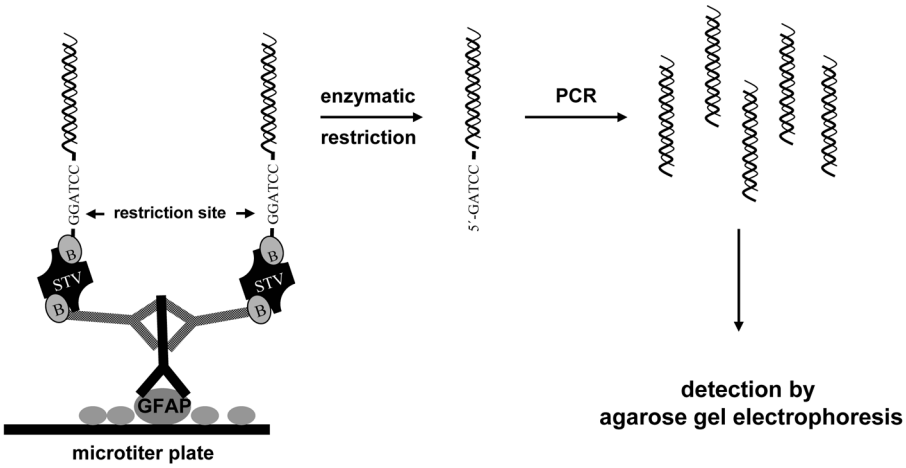

Figure 1. Schematic illustration of the immuno-PCR assay used in this study. GFAP, glial fibrillary acid protein; B, biotin; ST, streptavidin.

2.4 Visualisation and quantification of amplified PCR products

The amplified DNA was analysed by electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide-staining under UV light. Signal bands were scanned on a photo imager (Biorad, Munich, Germany) and quantified by densitometry using the Quantity One software (Biorad, Munich, Germany). For clear differentiation of background and positive signals the sensitivity settings of the image were adequately adjusted for each gel.

2.5 Enzyme-linked immunosorbent assay (ELISA)

Diluted protein suspensions were coated onto microtiter plates (Nunc Maxisorb, Roskilde, Denmark) at a volume of 50 µl/well. Washing and blocking procedures and antibody applications were done as described for the immuno-PCR assay. After removal of unbound antibodies, horseradish peroxidase-conjugated streptavidin was added in a concentration of 10 ng/ml for 30 min at room temperature. Stripes were finally washed and specific signals were visualised by colorimetric detection with a 3,3′,5,5′-tetramethylbenzidine (TMB) solution diluted in 0.05 M citrate buffer (pH 3.95) containing hydrogen peroxide (H_2O_2). After incubation for 15 – 30 min, the enzymatic reaction was stopped by addition of 1 M H_2SO_4. Plates were read at 450 nm on a Dynex microplate reader (VWR, Darmstadt, Germany).

3 Results and discussion

In the present study, an immuno-PCR assay is described for the ultrasensitive detection of GFAP. While this protein is highly expressed in astrocyte cells of the CNS, it is not found or found only in traces in peripheral nerves and organs [14]. Therefore, this protein is appropriate for detecting food contaminated with SRM material during or after the slaughtering processes, and consequently it is accepted as indicator protein for CNS tissues [14]. Hitherto, the detection of GFAP proteins in meats is performed using the ELISA techniques [14 – 15]. However, sensitivity is a limiting factor of these systems. Whereas the immuno-PCR approach is also primarily based on the ELISA technique, it is followed by powerful signal enhancement based on the combination of immunoreactivity with PCR-driven DNA amplification (Fig. 1). Antigenic proteins of homogenates bound on microtiter plates are captured by a polyclonal antibody specific for GFAP recognition. Biotinylated secondary antibodies are immunocomplexed and streptavidin is joined to the antigen – antibody complex. Streptavidin as a tetrameric protein can bind four biotins forming a bridge to the complex and biotin-labelled DNA. DNA is detached from the immuno-complex by enzymatic restriction, collected and finally amplified by conventional PCR as corresponding signal to the immunocomplex.

GFAP was determined by an immuno-PCR assay in brain suspensions at dilutions of $>5 \times 10^{-7}$ (Fig. 2A). Amplified DNA signals were visualised in agarose gels and quantified by densitometry. DNA signals resulted in decreasing intensities according to the antigen dilutions (Fig. 2B).

Because of the enhanced sensitivity of the immuno-PCR due to signal amplification, stringently configured specifications of the assay are necessary. The PCR settings, the amount of PCR template DNA and its presentation are considered to be critical conditions. Interfering proteins and high amounts of DNA template may prevent optimal DNA amplification resulting in low yields and nonreproducible results. To avoid interference with the PCR, DNA is detached from the immunocomplex and a *Bam*HI restriction site inserted into the biotin-labelled DNA sequence for enzymatic detachment. DNA is subsequently transferred and amplified by PCR. To balance high sensitivity and high

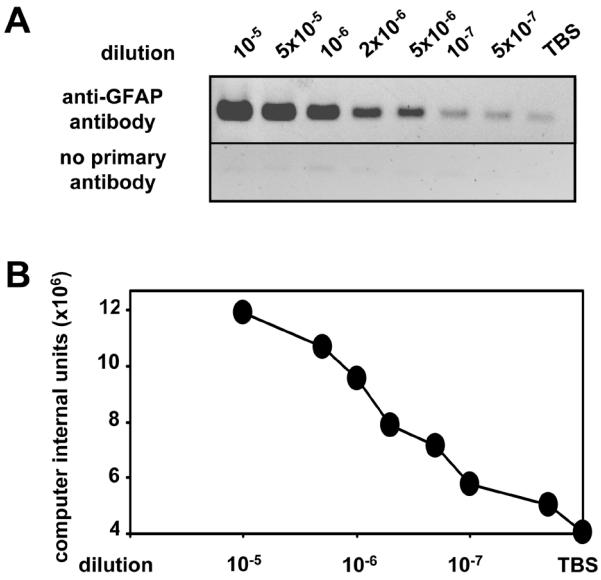

Figure 2. Detection and quantification of GFAP signals in brain homogenates. Proteins of brain homogenates (10%) were serially diluted in the range of 10^{-5}–5×10^{-7}. The marker protein GFAP was detected and signals were analysed by the immuno-PCR technique. (A) DNA immunocomplexed to GFAP proteins were amplified using conventional PCR. Signals were visualised after agarose gel electrophoresis. The negative control for background noise was dilution buffer without antigen (TBS) and the controls for true positive signals were identical approach conditions without application of specific anti-GFAP-antibodies for detection (-).(B) DNA amplicon signals were measured by densitometry and evaluated using the Quantity One software. Signal values are given as computer internal units ($\times 10^6$).

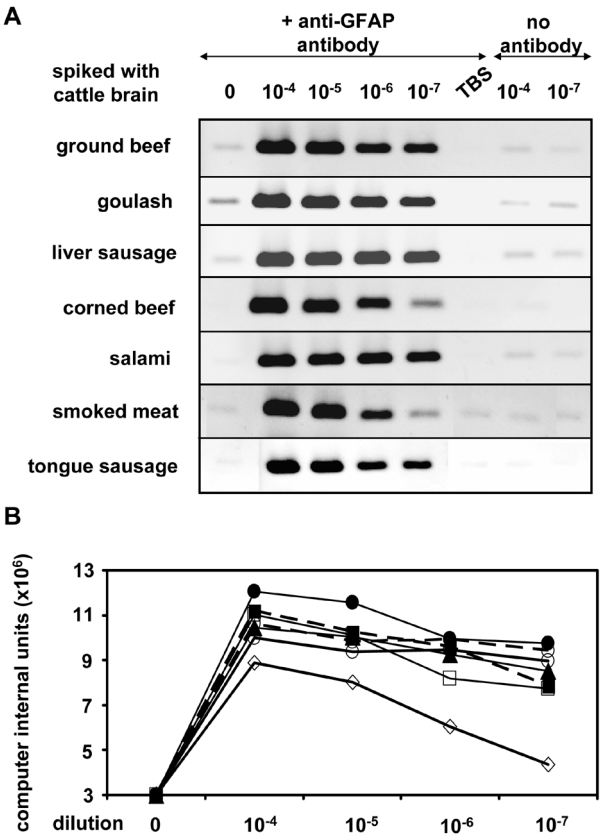

Figure 3. GFAP detection in meat products. Proteins of the meat products ground beef, goulash, liver sausage, corned beef, salami, smoked meat and tongue sausage were coated as pure product (0.01%) or spiked with cattle brain homogenates (10%) in the range of 10^{-4}–10^{-7}. GFAP proteins were detected by immunocomplexing with antibodies and DNA. (A) After enzymatic dissociation, DNA was amplified with conventional PCR (3 µl template and 26 cycles) and amplicons were visualised on agarose gels. Background noise was determined by dilution buffer without antigen (TBS) and background signals of pure meat products were identified (0). For specifity evaluation antigens were not allowed to bind to specific antibodies (no primary antibody). (B) Signals of the amplicons were quantified densitometrically using the Quantity One software. Intensities of DNA signals obtained from spiked meat and sausages are presented as computer internal units in the graph: ground beef (black circle), goulash (triangle), liver sausage (open circle), corned beef (square, dashed line), salami (open circle, dashed line), smoked meat (open rhombus), and tongue sausage (open square). The limit of background noise was defined at 3×10^6 units.

specificity, low amounts of template DNA (3–5 µl of a 50 µl volume) are amplified in order to find the concentration being optimal for PCR resulting high yields and reproducibility.

Nonspecific background signals may be caused by high concentrations of analysed proteins in complex suspensions of meat products and may occur during PCR when high numbers of amplification cycles are used. In the method we use, limiting the number of repeated runs to 26 was optimal when crude extracts were used. We achieve a 100 fold enhancement in sensitivity compared with an ELISA system under identical conditions (Table 1).

Apart from the amplification conditions, false-positive signals may also be caused by unspecific immuno-complexion. Specificity is evaluated by introduction of two controls: background noise of the assay is determined by adding buffer without antigen application and nonspecific binding of the secondary antibodies and biotinylated DNA are analysed by omitting the specific primary antibodies (Fig. 2).

In a further step, we have investigated the applicability of the assay for GFAP detection in meat and sausages. GFAP have been detected in various meat and sausage suspensions spiked with brain homogenates in dilutions of $>10^{-7}$ whereas no signals have been detected without spiking (Fig. 3A). Background signals may occur when meat homogenates with high protein concentrations are analysed. For this reason, meat suspensions are used in a concentration of 0.01%. Since a defined and objective differentiation between true and false-positive signals is impracticable for

Table 1. Detection of GFAP signals in meat products by immuno-PCR and ELISA.

Meat/sausage spiked with brain tissue[a)]	Method[b)]	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Negative controls[c)]
Brain	immuno-PCR	+[d)]	+	+	+	(+)[d)]	–[d)]
	ELISA	+	+	(+)	–	–	–
Ground beef	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Goulash	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Liver sausage	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Corned beef	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Salami	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Smoked meat	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	–	–	–	–
Tongue sausage	immuno-PCR	+	+	+	+	(+)	–
	ELISA	+	+	(+)	–	–	–

a) Proteins of meat and sausage products (0.01%) were spiked with cattle brain in serial dilutions based on 10% homogenates (10^{-4}–10^{-8}). Results are composed of three independent experimental set ups.
b) GFAP signals measured by ELISA were photometrically determined. DNA of the immuno-PCR assay was amplified with PCR and amplicons were visualised on agarose gels and signals were densitometrically quantified using the Quantity One software.
c) Negative controls are TBS buffer with no antigen application and food products without spiking.
d) +, positive signal (ELISA: OD >0.2; immuno-PCR: $>3 \times 10^6$ computer internal units); –, no signal; (+), positive in some samples.

the naked eye, DNA signals are computer-analysed and evaluated using the photo imager technique (Fig. 3B). Since background noise differs between gel runs, settings and values of sensitivity are adequately adjusted for each gel, thus, background signals have been reduced to values below the detection limit.

Miscellaneous modifications of the standard immuno-PCR technique, including real time immuno-PCR adaptations, have recently been developed for further improvement of this approach [22, 25, 29, 30- 33]. Whereas real time PCR is advantageous over traditional end-point methods in several aspects, the equipment and the accessory kits are expensive and, therefore, mostly restricted to specialised laboratories. The aim of this study was to develop a robust immuno-PCR assay available for general use in routine and diagnostic laboratories.

The signal enhancement achieved with the immuno-PCR was compared with the ELISA approach under identical conditions (Table 1). GFAP was detected in meat products spiked with brain homogenate at dilutions of $>10^{-5}$ using ELISA, whereas signals were achieved at $>10^{-7}$ brain homogenate dilutions by the immuno-PCR assay. In about one fifth to one fourth of our experiments, GFAP could be detected in dilutions of 10^{-8}. Hence, in comparison to the ELISA procedure, our results applying repeated runs demonstrated at least a 100 fold and sometimes a 1 000 fold enhancement in sensitivity.

The reproducibility of the immuno-PCR assay was demonstrated in repeated runs (Fig. 4) with various ground meat samples. Intensities of GFAP signals of ground meat spiked with cattle brain tissue were similar whereas unspiked meat displayed only light shadowy background signals.

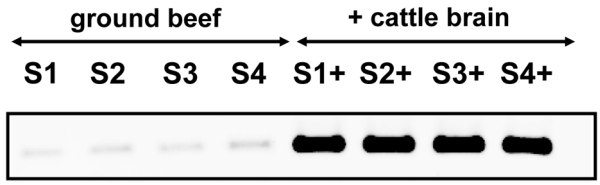

Figure 4. Reproducibility of the immuno-PCR assay. Four samples of ground beef were analysed for reproducibility of GFAP detection by the immuno-PCR approach. Proteins were coated as 0.01% homogenate (S1 – S4) or spiked with cattle brain (10^{-5} dilution of a 10% homogenate; S1 + to S4 +). DNA corresponding to the immuno complex was amplified by PCR (3 μl template DNA and 26 cycles). Signals were visualised on agarose gels under UV light after ethidium bromide staining. True positive signals are seen as dark black amplicon signals (S1 + – S4 +) with identical intensities whereas background signals may be seen as faint shadow lines (S1 – S4).

The detection of mRNA in GFAP-encoding sequences by real time RT-PCR is a highly sensitive measurement [18]. However, mRNA may be hydrolysed or fragmented during meat processing; thus, a technique based on the detection of stable protein seems to be more reproducible. Several immunological techniques have been described for detection of GFAP as a marker of CNS tissues in food products. However, most immunoassays are not able to discriminate between SRM and CNS tissues of other mammalian species as well as of younger ruminants which are not banned as

SRM. In this case, the use of species antibodies and/or the identification of animal species by species specific DNA hybridisation will provide data of the species processed in foods. Immunological commercial kits such as the Ridascreen Risk Material kit (R-Biopharm, Germany) have been studied [34]. Applying this kit, brain tissues could be detected in concentrations of up to 0.2%. Whereas the commercially available method is faster, the much higher sensitivity of the immuno-PCR approach – allowing the detection of traces of antigens – is a central aspect in regard to consumer protection. The capture of antigens by antibody-coated plates by the sandwich ELISA technique has the advantage of releasing interfering or blocking proteins and higher protein concentrations can be coated. However, the detection is limited by the affinity of the antibody to the antigen and the final conversion to visual signals obtained from the immuno complex. It is of particular interest, that a dramatic signal increase may be expected using a combination of both the capturing of the antigen by a sandwich ELISA followed by the signal amplification using the immuno-PCR approach.

4 Concluding remarks

The immuno-PCR method presented in this study is 100- to 1 000 fold more sensitive than the ELISA technique carried out under identical conditions, and this is of utmost importance for food safety and consumer protection against infections with prion diseases and transmission from cattle to humans. This technique was shown to be a robust and suitable tool in revealing trace amounts of GFAP as an indicator protein for brain in food imported via slaughtering and food-processing. After procedural standardisation and species specification, the immuno-PCR has a broad potential for applications in routine veterinary facilities, food industry and institutes carrying out food monitoring.

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