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Development of a Dot Blot Assay for the Rapid Detection of Central Nervous System Tissue on Meat and Contact Surfaces

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As a potential transmitter of bovine spongiform encephalopathy (BSE), tissue from bovine central nervous system (CNS) is not accepted in meat and meat products. Western blot analysis of the CNS marker myelin proteolipid protein (PLP) detects CNS contamination selectively and sensitively. In this study, a rapid dot blot assay using an anti-PLP antibody was developed to screen CNS contamination of meat and contact surfaces. The detection limit was 0.01% bovine brain in minced bovine muscle. When applied to a swab test, down to 0.5 mg of CNS tissue on meat or other surfaces was detectable. Other offal tissues or peripheral nerves did not interfere with the assay. The test allows a differentiation between mammalian and avian CNS but not among mammalian species. The swab test was applied immediately after slaughtering at several areas of the bovine head. CNS was not detectable at any region which may enter the food chain.

KEYWORDS: Bovine spongiform encephalopathy; central nervous system; meat; PLP; myelin proteolipid protein; dot blot; swab test

INTRODUCTION

Variant Creutzfeldt−Jakob disease is most likely transmitted by the consumption of tissues from cattle infected with bovine spongiform encephalopathy (BSE). The infectious agent, PrP^{sc}, is mainly detected in the central nervous system (CNS), whereas infection via muscle meat, blood, other organs, or milk is highly unlikely (*1*). Therefore, bovine CNS tissue, in particular brain and spinal cord, was defined by the EU as specified risk material and banned from human nutrition. Similar regulations were also passed in other countries, such as the United States, Canada, or Switzerland (*24*). On meat, CNS contamination is mainly caused by the slaughter process and is, therefore, located on the meat surface (*2*). To sausages or other meat products, CNS may be added as an ingredient to improve technological properties or as a component of advanced meat recovery products (*3, 4*).

Because of the potential health hazards of CNS and its possible presence in meat and meat products, analytical methods are required for its detection. Several markers for CNS in meat and meat products were suggested, such as cholesterol (*5*), neuron-specific enolase (NSE) (*5*), glial fibrillary acidic protein (GFAP) (*6*), or brain-specific fatty acids from sphingolipids (*7*). NSE has been analyzed by Western blot (*5*) and GFAP on the protein level by ELISA (*6*) and on the mRNA level by reversed transciptase PCR and gel electrophoresis or real time reversed transcriptase PCR (*8, 9*). Furthermore, a GC-MS method was developed to quantify fatty acids from sphingolipids (*7*).

Very recently, myelin proteolipid protein (PLP) was described as a novel marker for CNS contamination in food (*10*). PLP is a highly hydrophobic protein due to approximately 50% apolar amino acids and thioester linkage with long-chain fatty acids (*11*). Because of this hydrophobicity, PLP could be extracted selectively from the food matrix with an organic solvent. It was subsequently analyzed by Western blot using a polyclonal anti-PLP antibody. Due to the high and practically exclusive expression of PLP in CNS myelin, the PLP Western blot allowed a highly sensitive and selective detection of brain and spinal cord in meat and meat products (*10*). In the meantime, the assay was successfully applied to monitor brain adulteration of sausages on the German market (*3*). Although the method

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reliably and sensitively detects CNS contamination in meat and sausages, it is time-consuming and depends on some technical equipment. The purpose of this study was, therefore, to develop a faster method, which is easier to handle and does not rely on advanced analytical devices, using PLP as a marker protein for CNS contamination in meat and meat products.

MATERIALS AND METHODS

Reagents and Technical Equipment. Solvents (*n*-hexane, diethyl ether, and methanol) were purchased from Fisher Scientific (Schwerte, Germany). All other chemicals were purchased from Fluka (Buchs, Switzerland) and from Sigma-Aldrich (Taufkirchen, Germany). All chemical materials were of analytical reagent grade. Deionized water was distilled before use. Homogenization of sample material was carried out in PP-tubes (Greiner Bio-One, Solingen, Germany) with a T18 basic Ultra Turrax (Janke & Kunkel, Staufen, Germany). Swabs were purchased from Neolab (Heidelberg, Germany). The dot blotting apparatus (Minifold I), blotting paper (GB 003), and membranes (nitrocellulose Protran BA 85, pore size 0.45 μm) were obtained from Whatman/Schleicher & Schuell (Dassel, Germany). Polyclonal anti-PLP serum from rabbit was obtained from the Institute for Biochemistry, University of Erlangen, Germany.

Samples. Bovine tissue samples of different organs (brain, spinal cord, liver, lung, kidney, heart, spleen, thymus, and peripheral nerves) and brains of several avian species (duck, chicken, and turkey) and from mammals (cattle, goat, sheep, pork, and horse) were obtained from the Federal Research Centre for Nutrition and Food (BFEL) in Kulmbach, Germany. Minced bovine meat and other meat samples were purchased from local butcher shops. The swab tests of different parts of a cow head were taken 5–10 min after slaughtering in a local abattoir.

A quantity of 1.0 g of bovine brain was homogenized with an Ultra Turrax in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) to a total of 20 g. Aliquots of 10−200 μL of the resulting suspension were added to 10 g of bovine minced meat and thoroughly mixed by stirring, resulting in meat samples with CNS contents of 0.005%, 0.01%, 0.05%, and 0.1%. These mixtures were also used for the experimental determination of the detection limit.

For the analysis of species specificity, samples were prepared as follows: A quantity of 1 g of brain tissue of the different animal species was homogenized in TBS, which was added to a total of 5 g. Aliquots of 500 μL of the resulting suspensions were added to 10 g of bovine minced meat and thoroughly mixed to give samples with a CNS content of 1% (w/w). For some experiments, samples of pure homogenized brain were prepared.

Homogenized samples of different bovine tissues were analyzed directly to determine tissue specificity.

Samples for the swab test were prepared as follows: A quantity of 1.0 g of bovine brain was homogenized in TBS, which was added to a total of 20 g. Aliquots of 100, 40, and 20 μL of the resulting suspension, as well as 100 and 20 μL of the suspension diluted 1:10 in TBS, which are equivalent to 5, 2, 1, 0.5, and 0.1 mg of CNS, were dropped onto slices of beef or an aluminum plate. These samples were also used to determine the detection limit.

For the determination of tissue specificity, a homogenate of 10% of each bovine tissue in TBS was prepared, and an aliquot of 100 μL of each resulting suspension, equivalent to 10 mg of tissue, was dropped onto an aluminum plate.

To test storage stability, 1000 μL of a suspension of 1 g of bovine spinal cord in 20 g of TBS (equivalent to 50 mg of CNS tissue) was prepared as described above and dropped onto a beef slice. Several slices were prepared. The meat was stored at 0 °C for 5 days. Each day a swab was taken from one sample. A PLP extract was prepared immediately afterward. The extracts were stored at –21 °C before dot blot analysis.

Preparation of the PLP Extract. The PLP extract was prepared as described before (*10*). Briefly, a quantity of 5 g of sample material was extracted three times with at least 20 mL of *n*-hexane by repeated homogenizing (Ultra Turrax) and mixing. The resulting suspensions were filtered and the filtrates combined. The solvent was removed under reduced pressure. The residue was resuspended in 30 mL of diethyl ether to remove lipids and centrifuged at 3500 rpm for 10 min at 8 °C. The supernatant was discarded, and the resulting off-white pellet was dried and stored at –21 °C.

For dot blot analysis, 20 μg/μL PLP extract was dissolved in Laemmli sample buffer (2.5 mL of 0.5 M Tris-HCl, pH 8.5, 2.0 mL of glycerol, 400 mg of sodium dodecyl sulfate, 310 mg of dithiothreitol, 0.1 mg of bromphenol blue, and 10 mL of water) (*12*). This stock solution was kept at 4 °C until use.

Sample Preparation by Swab. The test suspensions were applied to the surfaces and allowed to dry for about 10 min. Then an area of 10 × 10 cm was wiped off with a cotton swab which had been soaked in *n*-hexane. The swab was thoroughly washed in 25 mL of *n*-hexane. The solution was filtered, and the filtrate was dried at 35 °C under reduced pressure. The residue was suspended in 1.5 mL of diethyl ether, transferred into a small centrifuge tube, and centrifuged for 30 s at 6400 rpm. The supernatant was discarded, and the resulting off-white pellet was dried and stored at −21 °C. The whole pellet was dissolved in 25 μL of Laemmli buffer for further analysis.

Dot Blot Analysis. For dot blot analysis, the PLP extract stock solution was diluted 1:400 or 1:50 (swab test) in concentrated solubilization buffer [4.0 g of sodium deoxycholate, 4 mL of 250 mM ethylenediaminetetraacetic acid (EDTA) in water, 4 mL of 250 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in water, 80 mL of methanol, and 200 mL with TBS, pH 7.4].

The solutions were thoroughly mixed, and aliquots of 200 μL were applied in each well to the nitrocellulose membrane using the dot blot apparatus. Each sample was dotted three times. One dot was used for analysis, one dot for Ponceau staining, and one dot as a negative control for unspecific coloration (see below).

The following incubation steps were performed at room temperature on a shaking device. The membranes were first washed for 5 min with water at room temperature to remove detergents and other soluble substances. The membrane was then cut in three pieces, each containing one spot of each sample. To test the homogeneity of the protein transfer, one membrane was stained with Ponceau solution (0.2% Ponceau S in 3% trichloroacetic acid) for 3–5 min on a shaking device. The excess of dye was removed from the membrane by washing with water. The other two membranes were blocked with TBS, pH 8.0, containing 5% skim milk powder for 60 min and afterward rinsed with TBS, pH 7.4. One of the blocked membranes was then incubated for 2 h with the polyclonal anti-PLP antiserum diluted 1:500 in the blocking buffer. The other one, serving as a control for unspecific coloration, was incubated only with blocking buffer. After three washing steps with TBS, pH 7.4, for 5 min each, the blots were incubated with a monoclonal alkaline phosphatase-conjugated goat−anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany; 0.6 mg/mL, diluted 1:5000 in blocking buffer) for 2 h. Then, the membranes were washed three times with TBS, pH 7.4, for 10 min each. The membranes were briefly rinsed with water. The binding of the antibodies to the target protein was visualized by a color reaction with the BCIP/NBT substrate system for alkaline phosphatase. To prepare the substrate solution, aliquots of 35 μL of BCIP solution (50 mg of 5-bromo-4-chloroindolyl phosphate dissolved in 1.0 mL of dimethylformamide) and 60 μL of NBT solution (50 mg of nitroblue tetrazolium chloride dissolved in 1.0 mL of 70% dimethylformamide) were added to 10 mL of substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, in water, pH 9.5) and mixed well. Both membranes were incubated with the freshly prepared substrate solutions for 3–5 min until a purple precipitate appeared. To stop the enzymatic reaction, the substrate solution was removed, and the blots were incubated with a solution of 20 mM Tris-HCl and 20 mM EDTA in water, pH 8.0, for 3 min. Afterward, membranes were rinsed with water and dried for storage. A purple precipitate which was stronger than the negative control (pure meat or swab from an uncontaminated surface) was considered as a positive signal.

RESULTS

Development of a Dot Blot Assay To Detect PLP in the Meat Matrix. The goal of this study was to develop a dot blot assay for the detection of CNS tissue in meat and on working

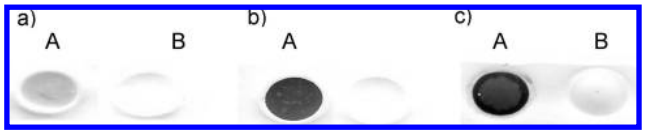

Figure 1. Detection of CNS contamination using a dot blot assay with anti-PLP antibody. Panel **a**: 0.1% of bovine brain in bovine minced meat (A) and bovine minced meat (B). Panel **b**: Swab test of 50 mg of bovine spinal cord on a beef surface (A) and of the uncontaminated beef surface (B). Panel **c**: Swab test of 100 mg of bovine brain on an aluminum plate (A) and of an uncontaminated plate (B). Representative blots from at least 15 replicate experiments are shown.

surfaces using a polyclonal antibody against PLP. Sample preparation was carried out as previously described for Western blot analysis: The meat samples were homogenized, PLP was extracted with *n*-hexane, and lipids were then removed by diethyl ether from the extract. For optimal signal to noise ratio, the resulting pellet was diluted 1:400 in solubilization buffer containing deoxycholate as detergent. A volume, equivalent to about 10 μg of PLP extract, was then applied to the dot blot device. Higher concentrations or higher volumes of the extract solution resulted in some background absorption of the negative control (minced meat) which led to a decrease of sensitivity. Immobilized PLP on the nitrocellulose membrane was detected with a polyclonal antibody. Other than in the Western blot assay, the PLP antigen was visualized by the alkaline phosphatase/BCIP/NBT system, resulting in a purple color which can be visually detected without technical equipment. A color which was stronger than the negative control (meat) was recorded as a positive signal.

Under these conditions, a concentration of 0.1% brain or spinal cord in minced meat gives a clear signal, whereas the meat matrix shows no color formation (**Figure 1a**).

Application of the Dot Blot Assay in a Swab Test. CNS contamination on meat is mostly caused by splattering during the slaughtering process and is, therefore, located on the meat surface. Thus, the dot blot assay was also applied to a swab test. **Figure 1b** shows the test result after the application of 50 mg of spinal cord on a slice of beef. Tests of uncontaminated meat did not show any coloration. The swab test was also successfully applied to contamination of CNS splatters on metal surfaces (**Figure 1c**).

Detection Limit. For the determination of the detection limit, aliquots of 0.005−0.1% bovine brain homogenate were added to minced beef. In all tests, a concentration of 0.01% CNS was still clearly detectable. Further, quantities of 0.1−5 mg of bovine brain homogenate were spread on slices of beef and an aluminum surface. After taking a swab, a contamination of 0.5 mg of brain homogenate on meat or aluminum could be easily differentiated from negative controls, for which a swab was taken from a slice of uncontaminated beef or a clean aluminum surface.

Tissue Specificity. In order to avoid false positive results from organs other than CNS, tissue specificity was tested with the dot blot assay. Thus, samples of pure bovine brain, heart, liver, spleen, peripheral nerve, kidney, thymus, and muscle (negative control), as well as a sample of 1% brain in meat, were extracted and subjected to the PLP dot blot assay (**Figure 2a**). Pure brain as well as 1% brain in minced meat showed clear coloration. Pure peripheral nerve showed a very faint signal, which was lower, however, than the signal of 1% brain in meat. No other tissues were recognized by the anti-PLP antibody. The experiment was also repeated with tissue contamination on surfaces. Quantities of 10 mg each of homogenized bovine spleen, liver, lung, heart, peripheral nerve, spinal cord, brain, and muscle were spotted on an aluminum plate. After drying, a swab was taken from the contaminated area, and PLP was measured in the dot blot assay. Under these conditions, only CNS material, which is brain and spinal cord, resulted in coloration (**Figure 2b**). The tests of all other tissues, including the peripheral nerve, were negative.

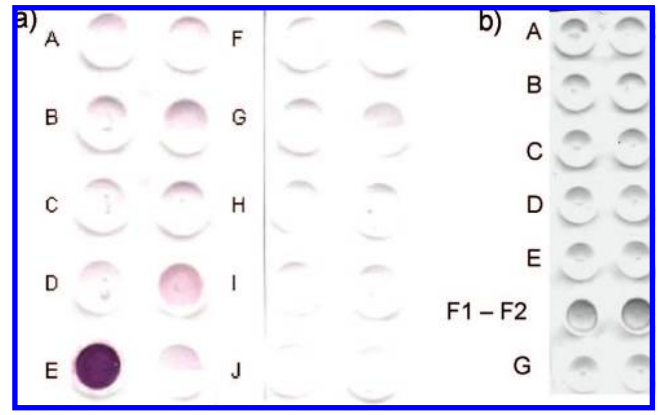

Figure 2. Tissue specificity of the dot blot assay using an anti-PLP antibody. In panel **a**, the pure bovine tissues heart (A), liver (B), spleen (C), peripheral nerves (D), brain (E), lung (F), kidney (G), thymus (H), and muscle (J) as well as 1% bovine brain in minced meat (I) were applied after PLP extraction. The right side of panel **a** shows the negative control where the same samples were analyzed in the same way but in the absence of the anti-PLP antibody. In panel **b**, a quantity of 10 mg of the bovine tissues spleen (A), liver (B), lung (C), heart (D), peripheral nerve (E), spinal cord (F1), brain (F2), and none (G) was spotted on a beef surface and a swab was taken, followed by PLP dot blot analysis. Representative blots from two replicate experiments are shown for panel **b** (A, C, D). All other experiments were performed in triplicate.

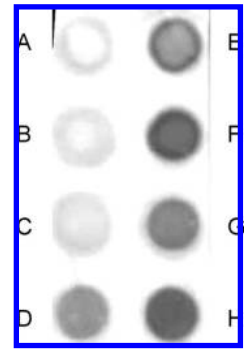

Figure 3. Species specificity of the dot blot assay using an anti-PLP antibody. A PLP extract was prepared from brain tissues from chicken (A), duck (B), turkey (C), sheep (D), horse (E), goat (F), pork (G), and cattle (H) and subjected to dot blot analysis. A representative blot from four replicate experiments is shown.

Species Specificity. To test species specificity, pure brain homogenates of chicken, duck, turkey, sheep, horse, goat, pork, and cattle were extracted and analyzed by the dot blot assay (**Figure 3**). In general, mammalian brain gave a very distinct color signal, whereas avian brain showed only very weak signals. Similar results were obtained when the brain homogenate (1%) was added to minced meat (data not shown).

Storage Stability. To test whether the time span between contamination and swabbing influences the detection of CNS, quantities of 50 mg of bovine brain homogenate each were applied to several beef slices. The meat was stored at 0 °C for 5 days. Each day, a swab was taken from one sample and extracted. At the end of day 5, the stored extracts were subjected to dot blot

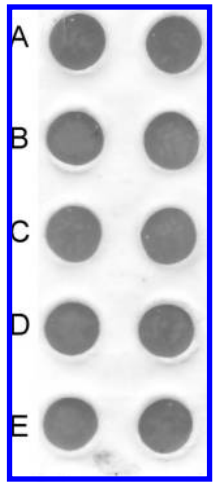

Figure 4. Storage stability of CNS contamination as assayed by the PLP dot blot. A quantity of 50 mg of bovine spinal cord each was spotted on beef slices, and a swab was taken after 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E) days. The test was performed twice.

analysis. **Figure 4** shows that the swab test can be taken even 5 days after contamination without losing signal intensity.

Application of the Swab Test to Freshly Slaughtered Cattle. Working under practice conditions, swabs were taken immediately after slaughtering at several areas of the bovine head, which bear a risk of CNS contamination. In detail, these were the cheek meat, the area around the captive bolt aperture, and the occiput muscle (muscle at the back of the head), as well as (after removal of the breech) the foramen magnum [aperture through which the medulla oblongata (extension of the spinal cord) enters the skull vault]. A positive signal was only evoked from the sample of the foramen magnum, whereas all other samples showed negative signals (data not shown).

DISCUSSION

PLP, for which data bank analysis revealed the most selective expression in CNS (*13*), has been described as a highly selective marker for CNS contamination in meat and meat products (*10*). Due to its high hydrophobicity, it can be selectively enriched by organic solvent extraction prior to Western blot analysis. This method provides high reliability and sensitivity and was suitable to screen 152 sausages on the German market for the addition of CNS material (*3*). The process, however, is time-consuming. The purpose of this study was, therefore, to develop a fast method for the detection of CNS contamination based on an immunochemical detection of PLP. The hydrophobic nature of PLP excludes the application of an enzyme-linked immunosorbent assay (ELISA), but dot blot assays have been successfully applied for quick and reliable analysis of hydrophobic membrane proteins (*14*). For the dot blot analysis, the PLP-containing extract is directly applied on a membrane and labeled with the anti-PLP antibody. Instead of using a secondary antibody labeled with a fluorescent dye, which has been detected by a phosphoimager, the secondary antibody was labeled with alkaline phosphatase. This tag allows visual detection of a purple color following the addition of the substrate and does not rely on expensive instrumental equipment. Visual detection is an important feature of the assay system. On the other hand, visual detection excludes statistical data analysis. In order to ensure the validity of experiments, several means were applied. First, all experiments were performed in replicates as indicated in the figure legends. Critical experiments, such as the determination of the detection limit or tissue specificity, were additionally repeated in a blinded way. For this purpose, the dot blots were blinded after preparation and then interpreted by a second independent person. Finally, in parallel experiments, analogous samples were analyzed by Western blot with fluorescence detection. In this setting, an imaging device was used for detection, and statistical data analysis was performed (*10*), supporting the present data.

Table 1. Detection Limits of Different Assays for Analysis of CNS in Meat or Sausage

CNS marker	assay system	detection limit or cutoff level (%)	ref
PLP	dot blot	0.01	present study
PLP	Western blot	0.025	*10*
NSE	Western blot	0.25, 0.5[a]	*16, 17*
GFAP	ELISA	0.2, 0.5[a]	*16, 17*
GFAP mRNA	reversed transcriptase PCR	0.01,[b] 0.25[a]	*8, 9*
fatty acids from sphingolipids	GC-MS	0.1−0.5	*18*

[a] In sausage matrix. [b] Because of cross-reactivity with PNS, a cutoff level of 0.1% was suggested.

The dot blot assay was first optimized to detect CNS contamination in minced meat as well as on surfaces of meat or metals. An important source of CNS tissue in ground beef is products from an advanced meat recovery system, when meat is mechanically detached from the spinal column (*4*). The presence of CNS tissue on meat surfaces derives from contamination by splattering during the slaughter process (*2*). Likewise, surfaces in the abattoir environment may come in contact with CNS tissue, bearing the risk of cross-contamination between infected and healthy carcasses. Therefore, every slaughterhouse in the EU, which uses head and cheek meat from cattle older than 12 months, is obliged to make spot tests (*15*). For the analysis of CNS contamination on meat or other surfaces, the dot blot assay was included into a swab test. The application of a swab assay accelerates sample preparation and avoids an interference of the meat matrix. Furthermore, it allows the monitoring of surfaces for CNS contamination, e.g. in the abattoir environment.

The assay clearly detects CNS tissue in minced meat or on surfaces (**Figure 1**). The detection limit of this assay for CNS in minced meat was 0.01%. Thus, the detection limit could even be decreased compared to a previously developed Western blot assay also using PLP as marker protein, where a concentration of 0.025% CNS was reliably detected (*10*). The improvement of sensitivity was probably achieved by labeling the secondary antibody with an enzyme-enhanced detection system instead of fluorescent dye. The detection limit was also excellent compared to other CNS detection assays (*8, 9, 16–18*) (see **Table 1**).

In order to avoid false positive results, high tissue specificity is required. Particular attention must be paid to tissue from the peripheral nerve system (PNS), because PNS is a legal and risk-free component of meat and meat products (*19*), and all of the known marker proteins for CNS also show some cross-reactivity with PNS (*4, 9, 20*). Using the Western blot assay, the polyclonal anti-PLP antibody did not detect PNS tissue (*10*). In PNS, DM-20 predominates, a developmentally regulated splice variant of PLP, which lacks amino acid positions 116−150 (*21*). The polyclonal anti-PLP antibody does not cross-react with DM-20, which can be explained by the fact that it was generated against the amino acid sequence 109−127 of PLP, partially

missing in DM-20. In the dot blot assay, only pure PNS tissue gave a weak signal which, however, was lower than the signal of 1% CNS in minced meat. On surfaces, 10 mg of PNS, an amount 20 times higher than the detection limit for CNS tissue, did not result in any coloration. No other tested tissues showed immunoreactivity for PLP. Therefore, false positive results due to other tissues than CNS are highly unlikely using the PLP dot blot assay.

Furthermore, species specificity of the dot blot assay was tested. The immunoreactivity of CNS from different mammalian species, such as cattle, pork, goat, sheep, and horse, was in a similar range. In contrast, avian brain samples from chicken, duck, and turkey gave only a weak signal. These results are in good accordance with those obtained by Western blot analysis using the same PLP antibody (*13*). The specificity can be explained by the change of two amino acids (^{120}Q → P and ^{125}S → A) in the epitope of mammalian and avian PLP.

The specified risk material includes mainly bovine and ovine CNS tissue from animals older than 24 months (*22*) or 30 months (*23*). Whereas immunochemical detection of GFAP and NSE is not able to distinguish between CNS tissues of different species (*16*), detection of GFAP mRNA (*9*) and fatty acids from CNS sphingolipids (*7*) shows species differences. However, it is highly unlikely that CNS contamination on meat, which is caused by splattering during the slaughter process, stems from species other than the meat matrix, so that species specificity is less important for this application than sensitivity and tissue specificity.

Finally, the applicability of the assay to samples from an abattoir was evaluated. First, it was investigated if the time period between CNS contamination and swab test has an influence on the test outcome. Up to 5 days of storage between contamination and swab test did not influence the results. Second, samples were taken from different sites on the head of cattle directly after slaughtering. The area around the captive bolt aperture, the occiput muscle, the foramen magnum, and cheek meat, which is used as food, were investigated in particular. As expected, only the sample from the foramen magnum induced a positive result, but no meat which is permitted for human nutrition.

In conclusion, the dot blot assay using an anti-PLP antibody proved to be a reliable method for the selective and specific detection of CNS contamination on meat and other surfaces.

ABBREVIATIONS USED

CNS, central nervous system; BSE, bovine spongiform encephalopathy; PrP^{sc}, prion protein; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; PLP, proteolipid protein; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BCIP, 50 mg of 5-bromo-4-chloroindolyl phosphate dissolved in 1.0 mL of dimethylformamide; NBT, 50 mg of nitroblue tetrazolium chloride dissolved in 1.0 mL of 70% dimethylformamide; PNS, peripheral nerve system.

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