

# Detection of PrP<sup>Sc</sup> in peripheral tissues of clinically affected cattle after oral challenge with bovine spongiform encephalopathy

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Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative prion disease that mainly affects cattle. Transmission of BSE to humans caused a variant form of Creutzfeldt–Jakob disease. Following infection, the protease-resistant, disease-associated isoform of prion protein (PrP<sup>Sc</sup>) accumulates in the central nervous system and in other tissues. Many countries have defined bovine tissues that may contain prions as specified risk materials, which must not enter the human or animal food chains and therefore must be discarded. Ultrasensitive techniques such as protein misfolding cyclic amplification (PMCA) have been developed to detect PrP<sup>Sc</sup> when present in minuscule amounts that are not readily detected by other diagnostic methods such as immunohistochemistry or Western blotting. This study was conducted to determine when and where PrP<sup>Sc</sup> can be found by PMCA in cattle orally challenged with BSE. A total of 48 different tissue samples from four cattle infected orally with BSE at various clinical stages of disease were examined using a standardized PMCA protocol. The protocol used brain homogenate from bovine PrP transgenic mice (Tgbov XV) as substrate and three consecutive rounds of PMCA. Using this protocol, PrP<sup>Sc</sup> was found in the brain, spinal cord, nerve ganglia, optic nerve and Peyer's patches. The presence of PrP<sup>Sc</sup> was confirmed in adrenal glands, as well as in mesenteric lymph nodes – a finding that was reported recently by another group. Interestingly, additional positive results were obtained for the first time in the oesophagus, abomasum, rumen and rectum of clinically affected cattle.

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## INTRODUCTION

Transmissible spongiform encephalopathies, or prion diseases, are invariably fatal neurodegenerative disorders associated with the accumulation of an abnormal isoform of the prion protein (PrP<sup>Sc</sup>), and include scrapie in sheep and goats, chronic wasting disease in deer and elk, Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cattle. Distribution of PrP<sup>Sc</sup> varies and depends primarily on the host species. In cattle,

prion infectivity is thought to be limited largely to the nervous system (Buschmann & Groschup, 2005a).

The BSE agent is transmissible to cattle (Hoffmann *et al.*, 2007; Wells *et al.*, 2007), exotic ungulates (Kirkwood *et al.*, 1990), cats (Pearson *et al.*, 1991; Eiden *et al.*, 2010) and humans (Bruce *et al.*, 1997; Hill *et al.*, 1997). The exact origin of BSE remains unclear, but the BSE epidemic was associated with oral exposure of cattle to prions through feedstuff contaminated with ruminant-derived proteins (Wilesmith *et al.*, 1988). To prevent such an event from recurring, many countries have instituted feed bans for

A supplementary figure is available with the online version of this paper.

ruminant proteins to be fed to cattle or other mammals. Moreover, the infection risk posed by incubating cattle is minimized by the removal of specified risk materials (SRMs) at slaughter to prevent tissues that may contain prions from entering the human food or animal feed chains. The SRM list includes tissues shown to contain BSE infectivity such as brain, spinal cord and intestine.

As new diagnostic techniques are developed and our knowledge of BSE expands, it is likely that the definition of SRM will continuously be revised. Immunohistochemistry and Western blotting fail to identify PrP<sup>Sc</sup> during the early incubation of prion diseases; however, highly sensitive transgenic mouse bioassays can detect prion infectivity in the jejunum, ileum and ileocaecal junction of cattle in the early months of infection (Hoffmann *et al.*, 2011). During the clinical phases of BSE, infectivity spreads centrifugally along nerve fibres into the periphery (Buschmann & Groschup, 2005a; Hoffmann *et al.*, 2011), and at the terminal stage of BSE, tissue from the tongue and nasal mucosa harbours infectivity, despite lacking detectable PrP<sup>Sc</sup> (Balkema-Buschmann *et al.*, 2011).

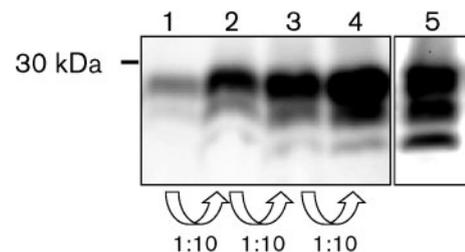
A promising approach for studying the PrP<sup>Sc</sup> distribution in infected animals is the protein misfolding cyclic amplification (PMCA) procedure (Saborio *et al.*, 2001). This technique relies on the binding of externally added cellular prion protein (PrP<sup>C</sup>) to abnormally folded PrP<sup>Sc</sup> seeds and its conversion/integration into larger protease-resistant (PrP<sup>res</sup>) fragments. These fragments can be disrupted by ultrasound into smaller fragments. Exponential PrP<sup>res</sup> growth can be achieved by serially applying binding/conversion and disruption cycles and can be used to detect trace amounts of PrP<sup>Sc</sup> seeds in samples.

This technique has been used widely for the amplification and detection of PrP<sup>Sc</sup> from hamsters (Saborio *et al.*, 2001), mice (Murayama *et al.*, 2007), bank voles (Cosseddu *et al.*, 2011), deer (Kurt *et al.*, 2007), sheep (Thorne & Terry, 2008), cheetahs (Eiden *et al.*, 2010) and humans (Jones *et al.*, 2007). In addition, PMCA has been used for the amplification of BSE-derived PrP<sup>Sc</sup> (Richt *et al.*, 2007; Balkema-Buschmann *et al.*, 2011). Recently, using a modified PMCA methodology, PrP<sup>Sc</sup> was found outside neuronal tissues in saliva, palatine tonsils, mesenteric lymph nodes, the ileocaecal region, adrenal glands and muscular tissues of BSE-affected cattle (Murayama *et al.*, 2010).

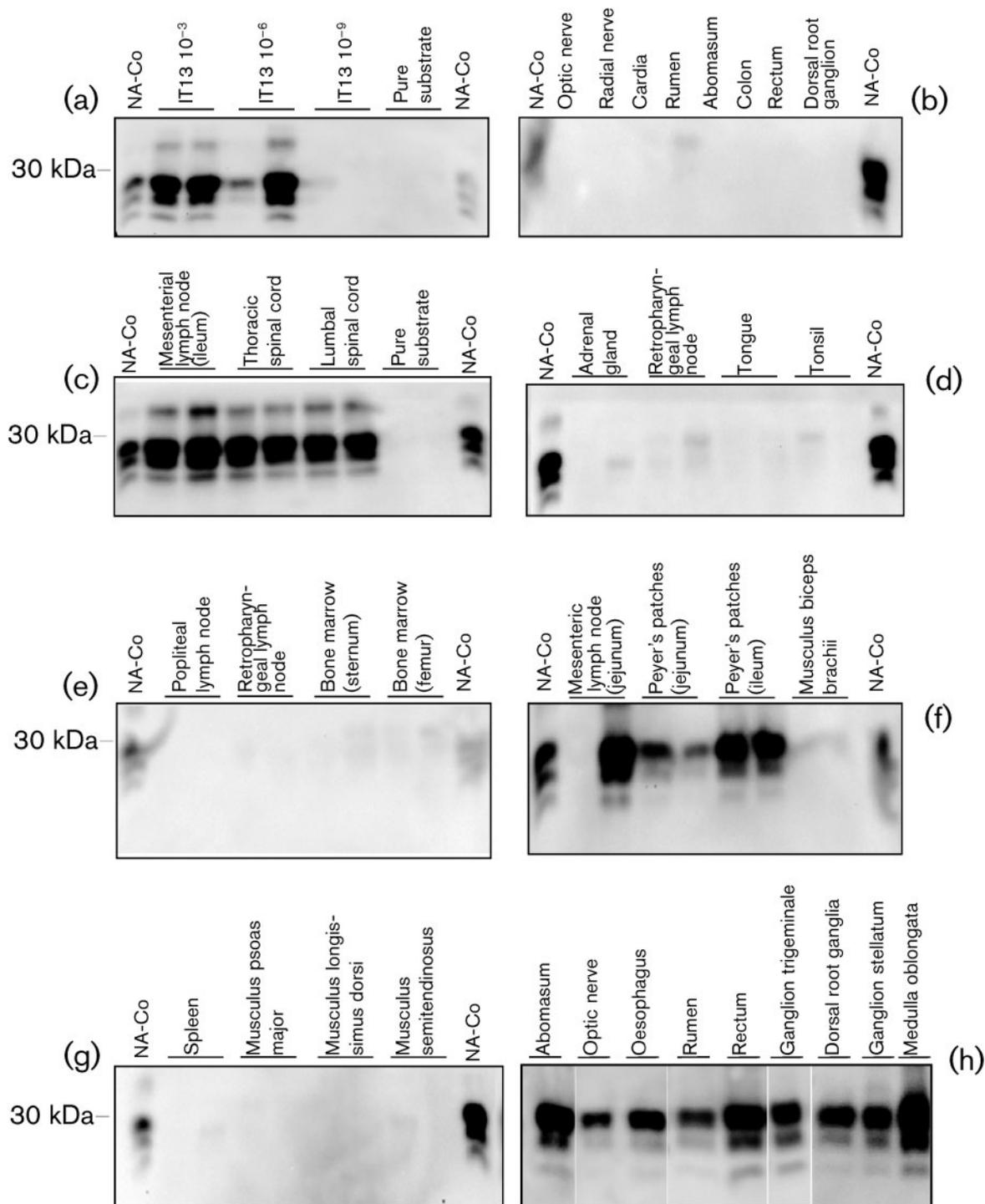
Here, we describe a PMCA study to detect PrP<sup>Sc</sup> in tissue samples collected during an oral BSE pathogenesis study (Hoffmann *et al.*, 2007). In total, 48 different tissues from clinically affected animals were examined to determine the spread of PrP<sup>Sc</sup> throughout the body. Using this approach, PrP<sup>Sc</sup> depositions were demonstrated in the central nervous system (CNS) and peripheral nervous system (PNS), but also in several, although not all previously reported, extraneuronal tissues. Moreover, we detected PrP<sup>Sc</sup> in tissues located all along the digestive tract that have so far been considered to be unaffected by BSE prions.

## RESULTS

To demonstrate the principal sensitivity and specificity of the PMCA protocol used here, we seeded 10 % brain material from an experimentally BSE-challenged animal that was killed 40 months post-inoculation (p.i.) at a  $10^{-3}$  dilution into 10 % brain homogenates of bovinized transgenic Tgbov XV mice (Fig. 1). Following 144 cycles of PMCA, a significant and specific amplification of protease-resistant prion protein was observed (Fig. 1, lane 2). The evolved PrP aggregates were designated PrP<sup>res</sup> because of their partial resistance to proteinase K (PK). Serial PMCA, in which amplified material was diluted tenfold into fresh Tgbov XV substrate and subjected to additional cycles of PMCA to increase the overall yield (Fig. 1, lanes 3–4), was carried out and indicated a template-assisted amplification of the original PrP<sup>Sc</sup> material. The magnitude of PrP conversion obtained by serial PMCA enabled the *in vitro* detection of minute amounts of PrP<sup>Sc</sup> in cattle tissues, which was demonstrated with serial BSE-positive control brain dilutions (Fig. S1, available in JGV Online). The serial PMCA was restricted to three rounds of dilution and amplification. When evaluating the round numbers of PMCA with non-infected Tgbov XV brain substrate or negative bovine tissue, *de novo* formation of PK-resistant PrP fragments was seen occasionally after five rounds. These PrP aggregates were biochemically indistinguishable from seeded PMCA reactions (data not shown). In order to avoid false-positive results, we therefore restricted our PMCA to three rounds. Every run in this study was carried out with pure substrate, as well as with eight negative-control samples derived from a mock-infected control cow (i.e. optic nerve, radial nerve, heart, rumen, abomasum, colon, rectum and dorsal root ganglion) (Fig. 2b) Under these conditions, *de novo* formation of PrP<sup>res</sup> was never observed.



**Fig. 1.** PMCA using PrP<sup>Sc</sup> seed from brain tissue collected from a BSE-challenged cow (IT13) collected at 40 months p.i. Tgbov XV mouse brain homogenate was used as the PrP<sup>C</sup> substrate. After a  $10^{-3}$  dilution, the sample was faintly positive by Western blotting prior to PMCA (lane 1). After one round of PMCA (144 cycles), a significant increase in PrP<sup>res</sup> was observed (lane 2). The product from round 1 was diluted 1 : 10 and subjected to a second round of PMCA (lane 3). The product was again diluted 1 : 10 and amplified by a third round of PMCA (lane 4). The original 10 % brain homogenate is included as a comparison (lane 5). All samples were PK digested and precipitated with phosphotungstic acid (PTA). Detection was carried out with mAb L42.



**Fig. 2.** PMCA analysis of tissue samples from an orally challenged bovine (IT49) at 36 months p.i. (a) Selected dilutions of the positive-control brain (IT13) homogenate loaded in duplicate wells:  $10^{-3}$ ,  $10^{-6}$  and  $10^{-9}$  dilutions and pure substrate. (b) Results for the negative-control samples from mock-inoculated cattle: optic nerve, radial nerve, heart, rumen, abomasum, colon, rectum and dorsal root ganglion. (c–h) Results for the mesenteric lymph node (ileum), thoracic spinal cord (T7), lumbar spinal cord (L3) and pure substrate (c) adrenal gland, retropharyngeal lymph node, tongue and tonsil (d), popliteal lymph node, retropharyngeal lymph node and bone marrow (femur) (e), mesenteric lymph node (jejunum), Peyer's patches (jejunum), Peyer's patches (ileum) and biceps brachii muscle (f), spleen, psoas major muscle, longissimus dorsi muscle and semitendinosus muscle (g) and abomasum, optic nerve, oesophagus, rumen, rectum, ganglion trigeminale, dorsal root ganglia, ganglion stellatum and medulla oblongata (h). All samples were digested with proteinase K and precipitated with PTA. Detection was carried out using mAb L42. NA-Co, Non-amplified control (10% homogenate from positive BSE control not subjected to PMCA, PK digested and PTA precipitated).

PMCA was performed on 48 tissues collected from cattle euthanized at 36, 40, 44 and 50 months after oral challenge with BSE (Table 1). All four animals exhibited clear clinical symptoms of a BSE infection. After the PMCA procedure, samples were digested with PK and precipitated with PTA prior to Western blotting. Each run was performed with a set of positive- and negative-control samples, and all tissue samples from challenged cattle were tested in duplicate. A complete run with samples of the cow euthanized at 36 months p.i. (IT49) is shown in Fig. 2(a-h). Each Western blot displays a non-amplified PrP<sup>Sc</sup> control (NA-Co) from a confirmed BSE field case (PK digested and PTA precipitated) loaded on the outside lanes as a control for PMCA-generated PrP<sup>res</sup> aggregates. Fig. 2(a) shows positive controls with seeds from positive-control brain tissue at dilutions of 10<sup>-3</sup>, 10<sup>-6</sup> and 10<sup>-9</sup>. The pure substrate revealed no PrP<sup>res</sup> aggregates. In Fig. 2(b), eight different tissue samples from mock-infected cattle were tested and remained negative. Positive results were obtained for samples from mesenteric lymph node (ileum), thoracic spinal cord and lumbar spinal cord (Fig. 2c). In addition, mesenteric lymph node (jejunum) and Peyer's patches from jejunum and ileum were positive (Fig. 2f). Other tissues were negative (Fig. 2d-g). Additional tissues detected as positive at this time point were the abomasum, optic nerve, oesophagus, rumen, rectum, nerve ganglia (trigeminal, dorsal root and stellatum) and medulla (Fig. 2h). In the animal euthanized at 40 months p.i. (IT13), PrP<sup>Sc</sup> was detected in nerve ganglia (dorsal root, coeliac and mesenteric, stellatum, trigeminal), medulla oblongata, Peyer's patches (ileum), optic nerve, adrenal gland and spinal cord (lumbar and thoracic) (Fig. 3a). In the animal euthanized at 44 months p.i. (IT22), PrP<sup>Sc</sup> was detected in the nerve ganglia (dorsal root, coeliac and mesenteric), medulla oblongata, Peyer's patches (jejunum and ileum), colon ascendens, optic nerve, rectum and spinal cord (lumbar and thoracic) (Fig. 3b). Most interestingly, PrP<sup>Sc</sup> was detected in the lowest number of tissues in the animal euthanized at 50 months p.i. (IT18), and comprised the coeliac and mesenteric ganglion complex, medulla oblongata, Peyer's patches (jejunum), rectum and spinal cord (lumbar and thoracic) (Fig. 3c).

In summary, all samples from the brain and spinal cord were positive. In three of the four analysed animals, samples from the dorsal root ganglia, coeliac ganglion, ileal Peyer's patches and optic nerve were positive. Interestingly, tissues from the rectum also were PrP<sup>Sc</sup> positive in three of the four animals examined. Stellate and trigeminal ganglion tissues and Peyer's patches from the jejunum were positive from two cattle, whereas positive results from the oesophagus, rumen, abomasum, mesenteric lymph nodes of the ileum and jejunum, adrenal gland and Peyer's patches of the colon were only positive in the animal killed at the earliest time point (36 months p.i.). The results for all tested tissues are summarized in Table 1 and demonstrate, for all four animals, PrP<sup>Sc</sup> deposition in the PNS, as well as the GIT. In one animal (IT49), PrP<sup>Sc</sup> was also found in the

lymphoreticular system, and in one case (IT13), the adrenal gland was affected.

## DISCUSSION

A total of 48 different tissue samples from four infected cattle of the German pathogenesis study were examined by a standardized PMCA protocol using substrate from transgenic mice overexpressing bovine PrP<sup>C</sup>. The eightfold overexpression of bovine PrP<sup>C</sup> in transgenic mouse brain provided the assay with an elevated level of PrP substrate that promoted PrP amplification but also favoured the risk of spontaneous aggregate formation *in vitro*. In order to exclude self-priming of PrP aggregates, several precautions were taken. Firstly, the assay was restricted to three amplification rounds to avoid the spontaneous PrP misfolding that we observed occasionally after five rounds of PMCA. The potential *de novo* formation of PK-resistant aggregates after extended consecutive PMCA rounds has also been reported by others (Saá *et al.*, 2006). Secondly, we omitted the use of any further reaction-enhancing additives, such as poly-anions (Thorne & Terry, 2008), heparin (Yokoyama *et al.*, 2011), glass beads (Pritzkow *et al.*, 2011) and Teflon beads (Gonzalez-Montalban *et al.*, 2011a).

The analytical sensitivity of published PMCA assays compared with mouse bioassays was significantly higher, for example by a factor of  $4 \times 10^3$  in the case of hamster scrapie (Saá *et al.*, 2006) or even  $10^5$  in the case of chronic wasting disease (Johnson *et al.*, 2012). In a recent publication, a PMCA assay for cattle-derived BSE was established in the presence of sulfated dextrane compounds, which displayed a  $10^5$ -fold higher sensitivity compared with a mouse bioassay (Murayama *et al.*, 2010).

However, we excluded additives in our reaction in order to minimize potential non-specific or opposing effects during PMCA, as additives can modulate PrP<sup>Sc</sup> fragmentation, the interaction with tissue-specific co-factors such as RNA or the availability of PrP<sup>C</sup> substrate, as described previously (Gonzalez-Montalban *et al.*, 2011b).

This restriction led to an analytical sensitivity of the PMCA for BSE in our hands that was comparable to transgenic mouse bioassays, as shown previously. It allowed the detection of the 10<sup>-6</sup> dilution of a 10 % BSE-positive brain homogenate, for which the infectivity titre was determined to be  $10^{6.1}$  LD<sub>50</sub> g<sup>-1</sup> (Balkema-Buschmann *et al.*, 2011). In the case of brain-derived BSE prions, the PMCA therefore yielded a high correlation between infectivity and conversion efficiency. It should be noted, however, that PMCA can have a lower analytical sensitivity when extraneuronal tissue is assayed, as was shown recently (Balkema-Buschmann *et al.*, 2011).

PrP<sup>Sc</sup> deposition was demonstrated in the brain, thoracic and lumbar spinal cord, optic nerve, Peyer's patches from jejunum, ileum and colon, nerve ganglia (dorsal root ganglia, coeliac ganglion, stellate ganglion and trigeminal

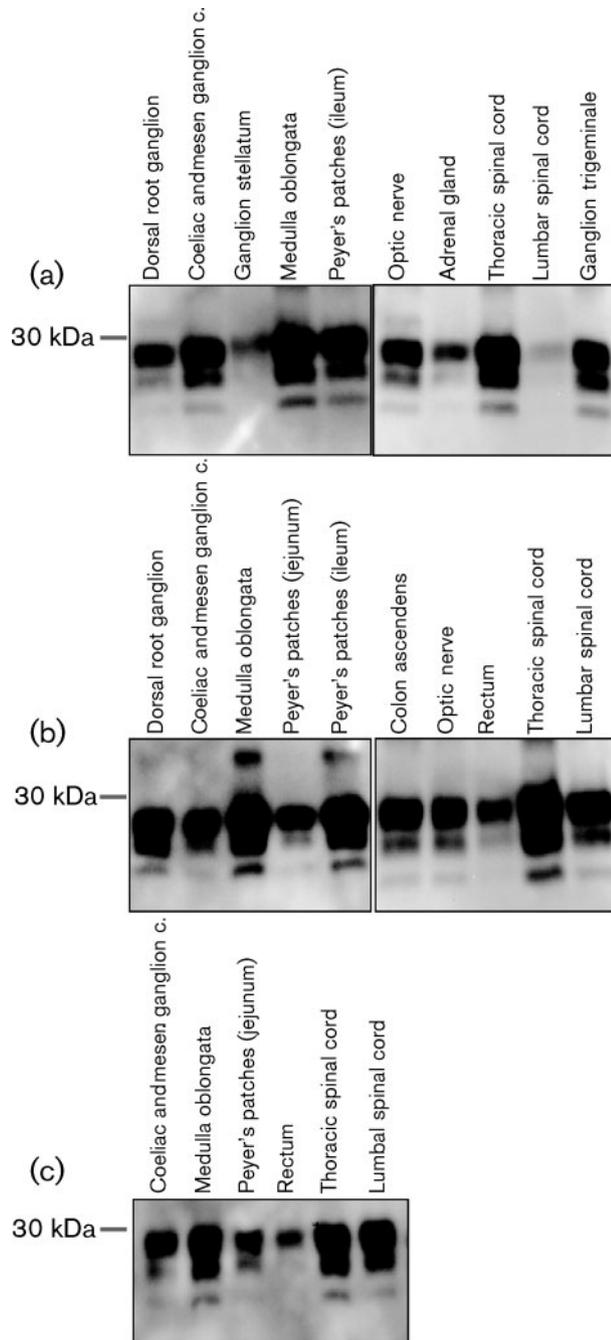
**Table 1.** Summary of PMCA analysis of tissues of infected cows from the pathogenesis study

+, Positive; -, negative; LRS, lymphoreticular system; GIT, gastrointestinal tract.

Site	Tissue	Cow IT49 (36 months p.i.)	Cow IT13 (40 months p.i.)	Cow IT22 (44 months p.i.)	Cow IT18 (50 months p.i.)
CNS	Medulla oblongata	+	+	+	+
	Thoracic spinal cord (T7)	+	+	+	+
	Lumbal spinal cord (L3)	+	+	+	+
PNS	Dorsal root ganglia	+	+	+	-
	Ganglion trigeminale	+	+	-	-
	Ganglion cervicale craniale	-	-	-	-
	Ganglion stellatum	+	+	-	-
	Coeliac and mesenterial ganglion complex	-	+	+	+
	Ganglion mesenteriale caudale	-	-	-	-
	Nervus opticus	+	+	+	-
	Nervus vagus (thorax/abdomen)	-	-	-	-
	Nervus facialis	-	-	-	-
	Nervus medianus	-	-	-	-
	Nervus radialis	-	-	-	-
	Nervus saphenus	-	-	-	-
	Nervus tibialis	-	-	-	-
LRS	Spleen	-	-	-	-
	Bone marrow (femur)	-	-	-	-
	Bone marrow (sternum)	-	-	-	-
	Retropharyngeal lymph node	-	-	-	-
	Tonsil	-	-	-	-
	Mediastinal lymph node	-	-	-	-
	Popliteal lymph node	-	-	-	-
	Mesenterial lymph node (jejunum)	+	-	-	-
	Mesenterial lymph node (ileum)	+	-	-	-
GIT	Tongue	-	-	-	-
	Salivary gland	-	-	-	-
	Oesophagus	+	-	-	-
	Rumen	+	-	-	-
	Abomasum	+	-	-	-
	Peyer's patches jejunum	+	-	+	+
	Peyer's patches ileum	+	+	+	-
	Colon ascendens (Ansa spiralis coli)	-	-	+	-
	Rectum	+	-	+	+
Muscles	Musculus longissimus dorsi	-	-	-	-
	Musculus psoas major	-	-	-	-
	Musculus biceps brachii	-	-	-	-
	Musculus semitendinosus	-	-	-	-
Other tissues	Heart	-	-	-	-
	Lung	-	-	-	-
	Liver	-	-	-	-
	Gall bladder	-	-	-	-
	Pancreas	-	-	-	-
	Kidney	-	-	-	-
	Adrenal gland	-	+	-	-
	Ovary	-	-	-	-
	Uterus	-	-	-	-
Mamma	-	-	-	-	

ganglion), adrenal gland and mesenteric lymph nodes. Interestingly, positive results were also obtained for the first time for the oesophagus, abomasum, rumen and rectum in one cow that was sacrificed at 36 months p.i. with clinical

BSE symptoms. These results demonstrated PrP<sup>Sc</sup> in tissues not previously detected by other techniques. Unusual tissues positive by PMCA implicate that PrP<sup>Sc</sup> is present but only in tiny amounts and that it is sparsely distributed. This may



**Fig. 3.** PMCA analysis of tissue samples from experimentally challenged cattle at 40, 44 and 50 months p.i. (a) Positive tissues from cow IT13 collected at 40 months p.i. comprising the nerve ganglia (dorsal root ganglion, coeliac and mesenteric ganglion complex and ganglion stellatum), medulla oblongata, Peyer's patches (ileum), optic nerve, adrenal gland, spinal cord (thoracic spinal cord and lumbar spinal cord) and ganglion trigeminale. (b) Positive tissues from cow IT22 collected at 44 months p.i. comprising the nerve ganglia (dorsal root ganglion and coeliac and mesenteric ganglion complex), medulla oblongata, Peyer's patches (jejunum and ileum) and colon ascendens, optic nerve, rectum and spinal cord (thoracic and lumbar). (c) Positive tissues from cow IT18 collected at 50 months p.i. comprising the coeliac and mesenteric ganglion complex, medulla oblongata, jejunal Peyer's patches, rectum and spinal cord (thoracic and lumbar). Only the third round of PMCA was subjected to SDS-PAGE. All samples were PK digested and PTA precipitated. Detection was carried out using mAb L42.

these results is that nerve endings could have been present in the material selected for PMCA, as submucosa and muscular layers contain enteric nerve components. Interestingly, for two of the animals presented here (IT49 and IT22), one of which displayed a wide PrP<sup>Sc</sup> distribution in the GIT, we were able in previous studies to reveal a clear PrP<sup>Sc</sup> accumulation in the enteric nervous system (ENS) of the ileum by immunohistochemistry (Hoffmann *et al.*, 2011; Kaatz *et al.*, 2012). From there, further dissemination along the network of the ENS is conceivable, although recent studies failed to detect PrP<sup>Sc</sup> in the ENS of the gut outside the ileum (Hoffmann *et al.*, 2011; Stack *et al.*, 2011). The results of the current study are supported by the fact that we previously detected infectivity in jejunal samples using a transgenic mouse bioassay (Hoffmann *et al.*, 2011). Whilst considering these positive results, it is important to note that *de novo* formation of PrP<sup>res</sup> was not detected in any of the control samples.

There was some variability in the tissues demonstrated to contain PrP<sup>res</sup> by PMCA. For example, the dorsal root ganglion and optic nerve samples were negative in the animal necropsied at 50 months p.i. (IT18) but were positive in the same tissues collected from animals necropsied at earlier time points. This is probably due to individual variation in the time course of PrP<sup>Sc</sup> dissemination from the gut to detectable amounts in the brainstem. Another obstacle in the detection of PrP<sup>Sc</sup> in cattle is the sometimes very limited amount that is present in different tissue samples, as recently shown by immunohistochemistry (Kaatz *et al.*, 2012). Even in animals that showed severe clinical signs and showed a strong CNS involvement, only individual cells or up to 20% of the cells may contain PrP<sup>Sc</sup> in a ganglion (Hoffmann *et al.*, 2011; Kaatz *et al.*, 2012). This sparse intensity is unique for BSE in cattle and is not comparable to the distribution pattern seen in classical scrapie in sheep and goats (van Keulen *et al.*, 2000; González *et al.*, 2009). Therefore, sampling from adjacent locations within the same tissue may explain the differing results of two individual analyses.

explain the lack of detection by immunological methods and the identification of PrP<sup>Sc</sup> only after amplification of the misfolded PrP. These tissues have only been bioassayed using low-sensitive wild-type mice in the past. Additional samples from body fluids (plasma, serum, cerebrospinal fluid and saliva) as analysed by others (Murayama *et al.*, 2010) were not included in this study.

Further work is required to pinpoint the exact source of PrP<sup>Sc</sup> in the gut wall in the samples from the oesophagus, abomasum, rumen and rectum. Samples used for PMCA were derived primarily from the mucosa and submucosa with the potential that muscular and connective tissue elements were also present. The most likely explanation for

Whilst most results in this study supported and expanded upon previous infectivity studies, we were unable to show PrP<sup>Sc</sup> accumulation in some tissues that have been described as infectious by others. For example, infectivity has been detected in the bone marrow and palatine tonsil (Wells *et al.*, 1999; Hamir *et al.*, 2011) in orally challenged cattle, but these tissues were devoid of detectable PrP<sup>Sc</sup> in any of the animals examined in this study. The positive results from tonsil were found in cattle examined at 10 months p.i. One reason why we did not detect any PrP<sup>Sc</sup> deposition in the tonsil samples may be that the presence of PrP<sup>Sc</sup> in the tonsils after oral challenge is transient. The same effect may be the reason why we only detected PrP<sup>Sc</sup> in samples from the GIT (oesophagus, abomasum, rumen and rectum) of the animal killed at 36 months p.i., whilst these samples were negative in the animals killed at later time points. To verify this, these samples collected from animals at different time points after inoculation will need to be examined. Another reason for the discrepancy between the detection of infectivity without detectable PrP<sup>Sc</sup> in the tonsils may be the fact that the bioassay performed in bovine PrP transgenic mice or in cattle is more sensitive than the PMCA, as shown recently for tongue and nasal mucosa (Balkema-Buschmann *et al.*, 2011). Furthermore, other mechanisms could be involved in infectivity, as PMCA is based on fragmentation and template-assisted amplification of PK-resistant PrP<sup>Sc</sup> fragments. This has been documented in previous publications, where prion diseases were caused by protease-sensitive forms of PrP<sup>Sc</sup> (Colby *et al.*, 2010) or by amyloid structures that are different from PrP<sup>Sc</sup> and that accumulate into atypical prion isoforms (Makarava *et al.*, 2012). Whether similar structures may also be present in the tissues of BSE-infected cows needs to be elucidated in future studies.

Finally, the sampling location may have influenced the results of these highly sensitive detection methods, as the accumulation of both PrP<sup>Sc</sup> and infectivity may occur in a highly restricted region of a tissue, for example in individual lymphoid follicles. It needs to be stressed that all examinations revealed negative results for peripheral lymph nodes and spleen, underlining previous findings that, with the exception of the gastrointestinal lymphoid tissue representing the portal of entry for the BSE agent, BSE pathogenesis in cattle completely bypasses the lymphoid system (Buschmann & Groschup, 2005b; Hoffmann *et al.*, 2007).

This study demonstrates an expansion in the scientific knowledge that should be considered for determining SRM regulations, namely, that BSE prions were demonstrated for the first time in the oesophagus, abomasum, rumen and rectum and may represent previously unrecognized risks in the transmission of BSE.

## METHODS

**Bovine samples.** The analysed samples were collected from cattle infected experimentally with BSE in a German BSE pathogenesis study (Hoffmann *et al.*, 2007, 2011) where each animal was challenged orally with 100 g brainstem material containing 10<sup>6.1</sup>

LD<sub>50</sub> g<sup>-1</sup> as determined in Tgbov XV mice (Buschmann & Groschup, 2005a). In addition to one mock-infected animal, four cattle killed between 36 and 50 months p.i., displaying clear clinical signs of BSE, a severe accumulation of PrP<sup>Sc</sup> and a severe spongiform encephalopathy in the obex region, were selected for in-depth analysis. The challenge experiments in cattle and mice described in this manuscript were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany, on the basis of national and European legislation, namely EU council directive 86/609/EEC for the protection of animals used for experiments.

**PMCA.** The PMCA protocol (Castilla *et al.*, 2005, 2008; Saá *et al.*, 2006) was applied with modifications (Eiden *et al.*, 2010; Balkema-Buschmann *et al.*, 2011). Brain tissue from Tgbov XV transgenic mice (Buschmann *et al.*, 2000) was used as the PrP<sup>C</sup> source (substrate) for the PMCA reaction. After CO<sub>2</sub>/O<sub>2</sub> euthanasia (Coenen *et al.*, 1995; Jones *et al.*, 1999), mice were exsanguinated and perfused with ice-cold PBS containing 5 mM EDTA prior to removal of the brain. Brain samples were homogenized to a concentration of 10% (w/v) in PMCA conversion buffer [PBS containing 150 mM NaCl, 1.0% Triton X-100, 5 mM EDTA and Complete Protease Inhibitor Cocktail (Roche)]. Homogenates were centrifuged at 1500 g for 30 s and the supernatant was immediately frozen at -70 °C.

The template for the positive-control PMCA reaction was a 10% (w/v) homogenate of bovine brain tissue in PMCA conversion buffer, which was stored at -20 °C until use. Serial dilutions were prepared in PMCA conversion buffer. Analyte tissue samples were also homogenized at 10% (w/v) in PMCA conversion buffer. A 10 µl aliquot of the analyte homogenate was suspended in 90 µl Tgbov XV brain substrate and transferred into 200 µl PCR tubes. As a negative control, samples collected from the same tissues of mock-infected control cattle were homogenized at 10% (w/v) in PMCA conversion buffer. As a positive control for each PMCA experiment, we used dilutions of a BSE-positive pool (10<sup>-3</sup>, 10<sup>-6</sup> and 10<sup>-9</sup> dilutions). The experiment was only considered valid if at least the 10<sup>-3</sup> and 10<sup>-6</sup> dilutions were identified as positive, with the negative control giving a negative result, as shown in Fig. 2.

The tubes were placed into a thin-walled adaptor and put on a microplate module of a sonicator (model 3000; Misonix). Samples were exposed to 144 cycles of sonication for 20 s each at a potency of 190–200 W (level 7), followed by a 30 min incubation. The reaction was carried out in a 35 °C incubator. After the first round of 144 cycles, samples were diluted 1:10 in fresh Tgbov XV brain homogenate and subjected to a second round of 144 cycles, which was again followed by a 1:10 dilution and a third PMCA round.

PMCA products were incubated with proteinase K (75 µg ml<sup>-1</sup>) for 60 min at 55 °C. After stopping the reaction with 0.5 mM PMSE, the samples were incubated with an equal amount of PBS/sarcosyl (4%, w/v) buffer for 30 min at 37 °C and subjected to PTA precipitation. The precipitation was carried out by incubation in 0.3% (w/v) PTA for 1 h at 37 °C with continuous shaking. After centrifuging for 30 min at 14000 g, the pellets were resuspended in 50 µl sample buffer and incubated at 95 °C for 5 min. Electrophoresis and immunoblotting using mAb L42 (R-Biopharm) were performed as described below for the cattle samples.

**PrP<sup>Sc</sup> detection by Western blotting.** Samples were analysed by SDS-PAGE using a 16% 0.75 mm thick acrylamide gel. The gels were transferred onto an Immobilon-P transfer membrane (Millipore) using a semi-dry blotting chamber. Membranes were incubated for 1 h in blocking solution composed of PBS containing 5% (w/v) low-fat milk and 0.1% (v/v) Tween 20 (PBST). Immunodetection of PrP<sup>Sc</sup> was conducted using mAb L42 at a concentration of 0.4 µg ml<sup>-1</sup> for 1 h at room temperature. Membranes were washed three times in PBST and incubated for 1 h with alkaline phosphatase-conjugated anti-mouse

secondary antibody, diluted in PBST to a final concentration of 0.15 µg ml<sup>-1</sup>. After three additional washing steps in PBST and two consecutive 2 min washing steps with assay buffer, detection was carried out using CDP-Star substrate (Roche) as the chemiluminescent substrate and an image analysis system (Versa Doc, Quantity One; Bio-Rad).

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## REFERENCES

- Balkema-Buschmann, A., Eiden, M., Hoffmann, C., Kaatz, M., Ziegler, U., Keller, M. & Groschup, M. H. (2011). BSE infectivity in the absence of detectable PrP<sup>Sc</sup> accumulation in the tongue and nasal mucosa of terminally diseased cattle. *J Gen Virol* **92**, 467–476.
- Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J. & other authors (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**, 498–501.
- Buschmann, A. & Groschup, M. H. (2005a). Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. *J Infect Dis* **192**, 934–942.
- Buschmann, A. & Groschup, M. H. (2005b). TSE eradication in small ruminants – quo vadis? *Berl Munch Tierarztl Wochenschr* **118**, 365–371.
- Buschmann, A., Pfaff, E., Reifenberg, K., Müller, H. M. & Groschup, M. H. (2000). Detection of cattle-derived BSE prions using transgenic mice overexpressing bovine PrP<sup>C</sup>. *Arch Virol Suppl* **16**, 75–86.
- Castilla, J., Saá, P., Hetz, C. & Soto, C. (2005). In vitro generation of infectious scrapie prions. *Cell* **121**, 195–206.
- Castilla, J., Gonzalez-Romero, D., Saá, P., Morales, R., De Castro, J. & Soto, C. (2008). Crossing the species barrier by PrP<sup>Sc</sup> replication in vitro generates unique infectious prions. *Cell* **134**, 757–768.
- Coenen, A. M., Drinkenburg, W. H., Hoenderken, R. & van Luijelaar, E. L. (1995). Carbon dioxide euthanasia in rats: oxygen supplementation minimizes signs of agitation and asphyxia. *Lab Anim* **29**, 262–268.
- Colby, D. W., Wain, R., Baskakov, I. V., Legname, G., Palmer, C. G., Nguyen, H. O., Lemus, A., Cohen, F. E., DeArmond, S. J. & Prusiner, S. B. (2010). Protease-sensitive synthetic prions. *PLoS Pathog* **6**, e1000736.
- Cosseddu, G. M., Nonno, R., Vaccari, G., Bucalossi, C., Fernandez-Borges, N., Di Bari, M. A., Castilla, J. & Agrimi, U. (2011). Ultra-efficient PrP<sup>Sc</sup> amplification highlights potentialities and pitfalls of PMCA technology. *PLoS Pathog* **7**, e1002370.
- Eiden, M., Hoffmann, C., Balkema-Buschmann, A., Müller, M., Baumgartner, K. & Groschup, M. H. (2010). Biochemical and immunohistochemical characterization of feline spongiform encephalopathy in a German captive cheetah. *J Gen Virol* **91**, 2874–2883.
- González, L., Martin, S., Sisó, S., Konold, T., Ortiz-Peláez, A., Phelan, L., Goldmann, W., Stewart, P., Saunders, G. & other authors (2009). High prevalence of scrapie in a dairy goat herd: tissue distribution of disease-associated PrP and effect of PRNP genotype and age. *Vet Res* **40**, 65.
- Gonzalez-Montalban, N., Makarava, N., Ostapchenko, V. G., Savtchenko, R., Alexeeva, I., Rohwer, R. G. & Baskakov, I. V. (2011a). Highly efficient protein misfolding cyclic amplification. *PLoS Pathog* **7**, e1001277.
- Gonzalez-Montalban, N., Makarava, N., Savtchenko, R. & Baskakov, I. V. (2011b). Relationship between conformational stability and amplification efficiency of prions. *Biochemistry* **50**, 7933–7940.
- Hamir, A. N., Kehrl, M. E., Jr, Kunkle, R. A., Greenlee, J. J., Nicholson, E. M., Richt, J. A., Miller, J. M. & Cutlip, R. C. (2011). Experimental interspecies transmission studies of the transmissible spongiform encephalopathies to cattle: comparison to bovine spongiform encephalopathy in cattle. *J Vet Diagn Invest* **23**, 407–420.
- Hill, A. F., Desbruslais, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J. & Lantos, P. (1997). The same prion strain causes vCJD and BSE. *Nature* **389**, 448–450, 526.
- Hoffmann, C., Ziegler, U., Buschmann, A., Weber, A., Kupfer, L., Oelschlegel, A., Hammerschmidt, B. & Groschup, M. H. (2007). Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy. *J Gen Virol* **88**, 1048–1055.
- Hoffmann, C., Eiden, M., Kaatz, M., Keller, M., Ziegler, U., Rogers, R., Hills, B., Balkema-Buschmann, A., van Keulen, L. & other authors (2011). BSE infectivity in jejunum, ileum and ileocaecal junction of incubating cattle. *Vet Res* **42**, 21.
- Johnson, C. J., Aiken, J. M., McKenzie, D., Samuel, M. D. & Pedersen, J. A. (2012). Highly efficient amplification of chronic wasting disease agent by protein misfolding cyclic amplification with beads (PMCAb). *PLoS ONE* **7**, e35383.
- Jones, D. M., Arters, J. & Berger-Sweeney, J. (1999). Carbon dioxide-induced anesthesia has no effect on brain biogenic amine concentrations in mice. *Lab Anim Sci* **49**, 316–318.
- Jones, M., Peden, A. H., Prowse, C. V., Gröner, A., Manson, J. C., Turner, M. L., Ironside, J. W., MacGregor, I. R. & Head, M. W. (2007). In vitro amplification and detection of variant Creutzfeldt–Jakob disease PrP<sup>Sc</sup>. *J Pathol* **213**, 21–26.
- Kaatz, M., Fast, C., Ziegler, U., Balkema-Buschmann, A., Hammerschmidt, B., Keller, M., Oelschlegel, A., McIntyre, L. & Groschup, M. H. (2012). Spread of classic BSE prions from the gut via the peripheral nervous system to the brain. *Am J Pathol* **181**, 515–524.
- Kirkwood, J. K., Wells, G. A., Wilesmith, J. W., Cunningham, A. A. & Jackson, S. I. (1990). Spongiform encephalopathy in an arabian oryx (*Oryx leucoryx*) and a greater kudu (*Tragelaphus strepsiceros*). *Vet Rec* **127**, 418–420.
- Kurt, T. D., Perrott, M. R., Wilusz, C. J., Wilusz, J., Supattapone, S., Telling, G. C., Zabel, M. D. & Hoover, E. A. (2007). Efficient in vitro amplification of chronic wasting disease PrP<sup>RES</sup>. *J Virol* **81**, 9605–9608.
- Makarava, N., Kovacs, G. G., Savtchenko, R., Alexeeva, I., Ostapchenko, V. G., Budka, H., Rohwer, R. G. & Baskakov, I. V. (2012). A new mechanism for transmissible prion diseases. *J Neurosci* **32**, 7345–7355.
- Murayama, Y., Yoshioka, M., Yokoyama, T., Iwamaru, Y., Imamura, M., Masujin, K., Yoshida, S. & Mohri, S. (2007). Efficient in vitro amplification of a mouse-adapted scrapie prion protein. *Neurosci Lett* **413**, 270–273.
- Murayama, Y., Yoshioka, M., Masujin, K., Okada, H., Iwamaru, Y., Imamura, M., Matsuura, Y., Fukuda, S., Onoe, S. & other authors (2010). Sulfated dextrans enhance in vitro amplification of bovine spongiform encephalopathy PrP<sup>Sc</sup> and enable ultrasensitive detection of bovine PrP<sup>Sc</sup>. *PLoS ONE* **5**, e13152.

- Pearson, G. R., Gruffydd-Jones, T. J., Wyatt, J. M., Hope, J., Chong, A., Scott, A. C., Dawson, M. & Wells, G. A. (1991).** Feline spongiform encephalopathy. *Vet Rec* **128**, 532.
- Pritzkow, S., Wagenführ, K., Daus, M. L., Boerner, S., Lemmer, K., Thomzig, A., Mielke, M. & Beekes, M. (2011).** Quantitative detection and biological propagation of scrapie seeding activity in vitro facilitate use of prions as model pathogens for disinfection. *PLoS ONE* **6**, e20384.
- Richt, J. A., Kasinathan, P., Hamir, A. N., Castilla, J., Sathiyaseelan, T., Vargas, F., Sathiyaseelan, J., Wu, H., Matsushita, H. & other authors (2007).** Production of cattle lacking prion protein. *Nat Biotechnol* **25**, 132–138.
- Saá, P., Castilla, J. & Soto, C. (2006).** Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. *J Biol Chem* **281**, 35245–35252.
- Saborio, G. P., Permanne, B. & Soto, C. (2001).** Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* **411**, 810–813.
- Stack, M. J., Moore, S. J., Vidal-Diez, A., Arnold, M. E., Jones, E. M., Spencer, Y. I., Webb, P., Spiropoulos, J., Powell, L. & other authors (2011).** Experimental bovine spongiform encephalopathy: detection of PrP<sup>Sc</sup> in the small intestine relative to exposure dose and age. *J Comp Pathol* **145**, 289–301.
- Thorne, L. & Terry, L. A. (2008).** In vitro amplification of PrP<sup>Sc</sup> derived from the brain and blood of sheep infected with scrapie. *J Gen Virol* **89**, 3177–3184.
- van Keulen, L. J., Schreuder, B. E., Vromans, M. E., Langeveld, J. P. & Smits, M. A. (2000).** Pathogenesis of natural scrapie in sheep. *Arch Virol Suppl* **16**, 57–71.
- Wells, G. A., Hawkins, S. A., Green, R. B., Spencer, Y. I., Dexter, I. & Dawson, M. (1999).** Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). *Vet Rec* **144**, 292–294.
- Wells, G. A., Konold, T., Arnold, M. E., Austin, A. R., Hawkins, S. A., Stack, M., Simmons, M. M., Lee, Y. H., Gavier-Widén, D. & other authors (2007).** Bovine spongiform encephalopathy: the effect of oral exposure dose on attack rate and incubation period in cattle. *J Gen Virol* **88**, 1363–1373.
- Wilesmith, J. W., Wells, G. A., Cranwell, M. P. & Ryan, J. B. (1988).** Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec* **123**, 638–644.
- Yokoyama, T., Takeuchi, A., Yamamoto, M., Kitamoto, T., Ironside, J. W. & Morita, M. (2011).** Heparin enhances the cell-protein misfolding cyclic amplification efficiency of variant Creutzfeldt–Jakob disease. *Neurosci Lett* **498**, 119–123.