BSE infectivity in the absence of detectable PrPSc accumulation in the tongue and nasal mucosa of terminally diseased cattle

Anne Balkema-Buschmann, Martin Eiden, Christine Hoffmann, Martin Kaatz, Ute Ziegler, Markus Keller and Martin H. Groschup

The pathogenesis of bovine spongiform encephalopathy (BSE) infections in cattle has been studied in recent years by using highly sensitive transgenic-mouse bioassays. It has been shown that in this species, the BSE agent amplifies almost exclusively in the central and peripheral nervous system. Even in animals that were killed in the clinical end stage of the disease, the lymphoreticular system was shown to be free of the infectious agent. No other animal species investigated to date exhibits such a restricted BSE-infectivity distribution pattern. However, there is growing evidence for a radial spread of infection from the central nervous system (CNS) into the periphery during the late stages of the disease. In this study, we challenged transgenic mice overexpressing the bovine prion protein with homogenates prepared from a wide variety of tissue samples collected from BSE-infected cattle. As prion infections involve the conversion of the cellular prion protein into its abnormally folded isoform (PrPSc), we applied various detection methods, such as the purification of scrapie-associated fibrils, immunohistochemistry, and the protein misfolding cyclic amplification technique. Despite negative results using these highly sensitive biochemical methods, we were, for the first time, able to detect BSE infectivity in the tongue and in the nasal mucosa of terminally diseased BSE field cases as well as experimentally challenged cattle by transgenic-mouse bioassay. This shows that BSE infectivity can be present in the peripheral tissues of terminally diseased cattle, including tissues used for human consumption.

INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease of cattle that was first observed in the UK in 1986 (Wells et al., 1987) and that has affected more than 180 000 animals in the UK since then. Indigenous BSE cases were also diagnosed in most European countries as well as in North America, Japan and Israel. Bovines were most likely infected by the oral uptake of BSE contaminated feed (Wilesmith et al., 1988). The transmission of BSE infectivity to man has caused a variant form of Creutzfeldt–Jakob disease (vCJD) in more than 200 humans, primarily in Great Britain but also in other European countries, Japan, North America and Saudi Arabia (Anonymous, 2010).

Upon oral uptake of the agent, the abnormally folded isoform of the prion protein (PrPSc) can first be detected in the ileal Peyer’s patches (PP) irrespective of the affected species (Heggebo et al., 2000; Terry et al., 2003; Lezmi et al., 2006), before it spreads towards the CNS. BSE-infected cattle display a unique pathway of infection because the spread of the BSE agent follows the parasympathetic and sympathetic nerve fibres of the autonomous nervous systems (Hoffmann et al., 2007), without involvement of the lymphoreticular system (LRS) apart from the gut-associated lymphatic tissues that represent the portal of entry for the BSE agent. Mouse bioassays using bovine-prion-protein (PrP) transgenic mice (Tgbov XV mice) revealed an essential restriction of BSE infectivity to the nervous system of bovines, while samples of the lymphoid (other than PP) and reproductive systems were devoid of BSE prions (Buschmann & Groschup, 2005). Recently, BSE infectivity has been detected in the palatine and lingual tonsil of BSE-infected cattle (Wells et al., 2005; Espinosa et al., 2007). Therefore these tissues must be removed from bovine tongues before they enter the human food chain. However, histological studies on the exact extension of lymphoreticular tissues in the tongue have shown that the rostral incision, which is currently practised, may still leave some solid tonsilar tissue attached to the tongue (Casteleyn et al., 2007).

In contrast, PrPSc and/or infectivity have been detected throughout the LRS of naturally and experimentally BSE- or scrapie-infected mice, rats, guinea pigs, hamsters and...
sheep (Manuelidis, 1975; Tateishi et al., 1980; van Keulen et al., 1996; Beekes & McBride, 2000; Andreoletti et al., 2000; Press et al., 2004). Moreover, PrPSc depositions have also been observed in the muscular tissues of the tongues of hamsters (Thomzig et al., 2004) and sheep (Casalone et al., 2005), as well as in other muscular samples from humans, sheep and mice (Bosque et al., 2002; Kovacs et al., 2004; Andreoletti et al., 2004).

A highly sensitive detection method for minute amounts of PrPSc has been published, which is based on the cyclic amplification of misfolded proteins (PMCA, protein misfolding cyclic amplification) (Castilla et al., 2005a, b; Saa et al., 2005). This method was adapted and optimized for the application presented in this publication.

We here describe the analysis of samples collected from preclinical and clinical BSE cases. Among these were samples from the nervous system, the tongue and the nasal mucosa. While immunohistochemistry, scrapie-associated fibrils (SAF) immunoblot and PMCA analysis were performed for the detection of PrPSc, the presence of infectivity was examined by mouse bioassay in Tgbov XV mice overexpressing bovine PrP. We considered it important to perform both the PrPSc analysis and the transgenic mouse bioassay for the tissues of interest, as interpretation of these results provide answers concerning the correlation of PrPSc accumulation and infectivity titre in the case of bovine BSE.

RESULTS

For a better comparison of the analytical sensitivity of the most sensitive methods for detecting either BSE infectivity (mouse bioassay in Tgbov XV mice) or PrPSc deposition (SAF immunoblot and PMCA), we analysed a serial dilution of the British brain-pool sample which had been used for the oral challenge of cattle (Hoffmann et al., 2007). The infectivity titre for this sample had been determined as \(10^{6.6} \text{LD}_{50} \text{g}^{-1}\) in the transgenic mouse model (Hoffmann et al., 2007). Using PMCA, we were able to demonstrate the presence of PrPSc up to a dilution of \(10^{-6}\), indicating a comparable sensitivity for these two methods. It must however be kept in mind that both methods aim at different targets, i.e. the mouse bioassay detects infectivity while PMCA detects PrPSc present in a given sample. In contrast, the SAF-immunoblot only enabled a weak detection of the dilution of \(10^{-4}\) (Fig. 1). Including the applied immunohistochemistry (IHC) techniques in this comparison was technically impossible.

Tissues from two BSE field cases in the UK were analysed. We first inoculated 10% homogenates into Tgbov XV mice in order to search for BSE infectivity. As expected, the brainstem, optic nerve, facial nerve, trigeminal ganglion, cervical cranial ganglion and the cervical ganglion from both animals contained BSE infectivity (Table 1). Infectivity loads were highest in samples from the brainstem, trigeminal ganglion and cervical cranial ganglion as can be deduced from the complete or almost complete attack rates in the inoculated mouse groups. The incubation times in the mice inoculated with these samples were relatively short: 288–326 days (brainstem), 305–338 days (trigeminal ganglion) and 367–391 days (cervical cranial ganglion) (Table 1). In correlation to earlier BSE titration experiments performed in Tgbov XV mice (Buschmann & Groschup, 2005), such incubation times were roughly deduced from the complete or almost complete attack rates in the inoculated mouse groups. The incubation times in the mice inoculated with these samples were relatively short: 288–326 days (brainstem), 305–338 days (trigeminal ganglion) and 367–391 days (cervical cranial ganglion) (Table 1). In correlation to earlier BSE titration experiments performed in Tgbov XV mice (Buschmann & Groschup, 2005), such incubation times were roughly estimated to be correlated with infectivity loads ranging between \(10^{4.4} \text{ID}_{50} \text{g}^{-1}\) (ID50 median infective dose) (~280 days) and \(10^{2} \text{ID}_{50} \text{g}^{-1}\) (~400 days). The lower attack rates and longer incubation times of mice inoculated with the optic nerve (505 / 457 days), facial nerve (480 / 492 days) and cervical ganglion samples (599 days and no disease) indicate infectivity loads below \(10^{2.5} \text{ID}_{50} \text{g}^{-1}\). A

Fig. 1. Analytical sensitivity of the applied methods for the detection of PrPSc and infectivity in a BSE brain pool. (a) Serial dilutions of a British BSE-positive brainstem pool were analysed using different biochemical tests and a mouse bioassay in Tgbov XV mice. For comparison, one of the most frequently used rapid tests for BSE surveillance (IDEXX HerdChek) was included in the assay. (b) Immunoblot displaying the analytical sensitivity of the SAF immunoblot on serial dilutions of the same brain pool. mAb L42 was used for detection. (c) PMCA of serial dilutions (\(10^{-3}\), \(10^{-5}\), \(10^{-7}\) and \(10^{-9}\)) of the British BSE-brainstem pool used in (a) and (b). First, second and third rounds of duplicate amplifications were loaded on three gels. After the third round of amplification PrPSc is still detectable at a dilution of \(10^{-9}\). This experimental set-up was included in each PMCA procedure as a positive control. The experiment was only considered valid if at least the \(10^{-3}\) and \(10^{-6}\) dilutions were identified as positive, with the negative control remaining negative. NA-Co, Not amplified control, a brain sample from the BSE-infected animal IT13 was used after phosphotungstic acid (PTA) precipitation and proteinase K (PK) digestion.

more precise determination of the infectivity loads of these samples is hampered by the fact that not all animals in the challenged groups succumbed to the disease (Table 1). Thus, an end-point titration of these tissues would be necessary to enable an exact quantification of infectivity.

Also, the mice challenged with samples from the nasal mucosa and the tongue, which had so far always been determined to be free of BSE infectivity, succumbed to the disease. Three of 13 mice and eight of 14 mice inoculated with the nasal mucosa developed BSE after incubation times of 466 and 492 days, respectively. After challenge with the tongue samples, seven of 11 mice and one of 10 mice succumbed to the disease after 498 and 728 days, respectively. These incubation times indicate the presence of infectivity loads of \(10^{2.5}\) ID\(_{50}\) g\(^{-1}\) in the corresponding tissues.

We were interested to know whether any PrP\(^{Sc}\) deposition in these samples was detectable. We used SAF precipitation, as this method had proven to have the highest analytical sensitivity in our hands. However, due to the limited material available, we were only able to analyse a small volume (200 \(\mu\)l) of a 10% homogenate for all samples, except for the tongue where a large-scale preparation starting with 2 g material was possible. Using this technique, we were unable to detect PrP\(^{Sc}\) in any of these tissues (Table 1, Fig. 2).

The second attempt to reveal PrP\(^{Sc}\) deposition in these tissues was by IHC by using two different highly sensitive monoclonal antibodies. To increase the probability of detecting a PrP\(^{Sc}\) deposition that might only be present in individual cells, we applied a serial cutting technique. By using this approach, we were able to detect PrP\(^{Sc}\) deposition in the trigeminal ganglion samples of both animals, but in no other nervous-tissue samples under analysis. Moreover, no PrP\(^{Sc}\) deposition was detected in any of the anatomical structures of the tongue or nasal mucosa taken from animals PG1282/00 and PG1283/00 (data not shown).

Samples were then examined by PMCA. We report here for the first time the amplification of BSE-PrP\(^{Sc}\) material using 10% brain homogenates of Tgbov XV mice as a substrate. These transgenic mice overexpress the non-pathogenic form of PrP (PrP\(^{C}\)), by a factor of eight in their brains as compared with normal cattle brain. Using a standard PMCA protocol including three consecutive rounds of PMCA, we were able to demonstrate highly efficient amplification of the transgenic PrP\(^{C}\) (Fig. 3a). After each 1 : 10 dilution in Tgbov substrate and a subsequent PMCA round, PrP\(_{res}\) fragments were generated at the same or even greater levels compared with the previous round, which indicates seed-derived amplification of PrP\(_{res}\) by an autocatalytic mechanism. No PrP\(_{res}\) fragments were generated in the absence of PrP\(^{Sc}\) seeds (Fig. 3a, lanes 7–9). Analysis of PMCA generated PrP\(_{res}\) fragments revealed a typical PK-resistant banding pattern of di-, mono- and unglycosylated PrP\(_{res}\) fragments. Using three consecutive PMCA rounds, we demonstrated PrP\(^{Sc}\) amplification up to

<table>
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<tr>
<th>Cattle no. and tissue</th>
<th>Biochemical analysis</th>
<th>Mouse bioassay</th>
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<tr>
<td></td>
<td>SAF immunoblot</td>
<td>PMCA</td>
</tr>
<tr>
<td>Pg1282/00 Brainstem</td>
<td>+ + +</td>
<td>+ +</td>
</tr>
<tr>
<td>Nervus opticus</td>
<td>–</td>
<td>+/−</td>
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<tr>
<td>Nervus facialis</td>
<td>–</td>
<td>–</td>
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<td>Trigeminal ganglion</td>
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<tr>
<td>Cranial cervical ganglion</td>
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<td>–</td>
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<td>Medial/caudal cervical ganglion*</td>
<td>–</td>
<td>–</td>
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<td>Nasal mucosa</td>
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<td>Tongue</td>
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<td>Pg1283/00 Brainstem</td>
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<td>+ +</td>
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<td>N. opticus</td>
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<td>ND</td>
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<td>N. facialis</td>
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<td>Trigeminal ganglion</td>
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<td>Cranial cervical ganglion</td>
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<td>Tongue</td>
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a 10⁻⁶ dilution of the bovine BSE brain pool (Fig. 1c). Serial dilutions (10⁻⁶–10⁻¹²) of British BSE-brainstem pool were subjected to three rounds of PMCA in duplicate. Amplified PrPres fragments were detected after the first, second and third rounds of PMCA (Fig. 1c). The standard procedure was restricted to three consecutive PMCA rounds only in order to avoid self priming and de novo PrPres formation which leads to false-positive results.

Using PMCA we could clearly verify PrPSc deposition in the brainstem of two clinically affected field cases designated BS1 and BS2 (Fig. 4a, lanes 2–5). Both samples were analysed in duplicate. No amplification was obtained from the cervical cranial ganglion (Fig. 4a, lanes 6–9).

To verify our results obtained from the BSE field cases, we applied the same analytical methods to samples collected from the tongue and nasal mucosa of cattle sacrificed during an oral BSE-pathogenesis study (Hoffmann et al., 2007). As described for the two BSE field cases, neither SAF immunoblot, IHC nor PMCA analyses were able to detect any PrPSc deposition in the tongue or nasal mucosa of the animals examined. When analysing tongue samples using PMCA, we could not detect any PrPres formation (Fig. 4b, lanes 2–9). Also no PrPres was detected in tongue samples from control animals (KT67, KT31 and KT30) (Fig. 3b, lanes 2–7). Negative results were also obtained from the...
nasal mucosa of BSE-positive animals from the BSE-pathogenesis study (Fig. 4b, lanes 12–19). The PMCA procedure was validated with BSE brain-pool samples in corresponding dilutions (Fig. 1). All samples were tested in duplicate. We then performed a PMCA spike experiment using a BSE-positive brain sample with and without addition of a lingual muscle homogenate to exclude any inhibiting effect through the analysis of tongue samples. No reduction in signal intensity was observed for the sample spiked with lingual muscle (Fig. 4c, lanes 2–5) as compared to the BSE-positive brain sample alone (Fig. 4c, lanes 6–9). Even after two additional rounds of PMCA, no differing results were obtained (not shown).

Homogenates prepared from the tongue samples from the experimentally challenged bovines that had been found negative for PrPSc deposition were then used for transgenic-mouse bioassays. Infectivity was easily detected in samples from cattle showing clinical symptoms of a BSE infection, i.e. animals that were sacrificed 32 months after oral challenge. These mouse challenges resulted in 30–92% attack rates and incubation times that ranged between 386 and 523 days (Table 2). According to these results, the tongue of clinically BSE-affected animals contained infectivity loads between $10^{2.3}$ and $10^3$ ID$_{50}$ g$^{-1}$, which is in the same range as the titre determined for the facial nerve, optic nerve and cervical ganglion samples from the two clinical BSE field cases, and for the tongue from animal PG1282/00.

**DISCUSSION**

This manuscript describes the highly sensitive analysis of different neuronal and non-neuronal samples from BSE-infected cattle. As estimated from the incubation...
Table 2. Analysis of tongue and nasal-mucosa samples from animals orally challenged with BSE by using biochemical methods and mouse bioassay in Tgbov XV mice

Clinical status: 0, no clinical signs; 1, weak and unspecific neuronal symptoms; 2, clear neurological symptoms, suspicious of a BSE infection; 3, strong neurological symptoms most probably associated with BSE, animals had to be sacrificed within a few days. Immunohistochemical and biochemical analysis: –, Negative; +/-, inconclusive; (+), very weak positive; +, weak positive; ++, moderate positive; ++++, strong positive; ND, not done. *, Diseased/inoculated animals and incubation times in days (SEM). TB, Tongue body.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Months p.i.</th>
<th>Clinical status</th>
<th>PrPSc obex by IHC</th>
<th>Tongue IHC</th>
<th>SAF immunoblot</th>
<th>PMCA</th>
<th>Bioassay in Tgbov XV</th>
<th>Nasal mucosa</th>
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<tbody>
<tr>
<td>IT 44</td>
<td>1</td>
<td>0</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>IT 19</td>
<td>4</td>
<td>0</td>
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<td>–</td>
<td>ND</td>
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<tr>
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<td>ND</td>
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<tr>
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<td>12</td>
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<td>–</td>
<td>–</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>IT 46</td>
<td>16</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>0/9 &gt;647 days</td>
<td>–</td>
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<tr>
<td>IT 10</td>
<td>20</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>IT 26</td>
<td>24</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>0/9 &gt;731 days</td>
<td>–</td>
</tr>
<tr>
<td>IT 08</td>
<td>28</td>
<td>0 (+) 1 neuron</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>0/12 &gt;728 days</td>
<td>–</td>
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<tr>
<td>IT 43</td>
<td>32</td>
<td>2</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5/15 494 days (32.14)</td>
<td>–</td>
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<tr>
<td>IT 49</td>
<td>36</td>
<td>3</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5/8 523 days (34.63)</td>
<td>–</td>
</tr>
<tr>
<td>IT 13</td>
<td>40</td>
<td>2–3</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>13/14 390 days (9.81), TB:11/15 396 days (20.56)</td>
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<tr>
<td>IT 15</td>
<td>44</td>
<td>3</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>11/12 437 days (25.47)</td>
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<tr>
<td>IT 64</td>
<td>47</td>
<td>3</td>
<td>++++</td>
<td>–</td>
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<td>–</td>
<td>8/12 484 days (28.74), TB: 8/13 409 days (15.77)</td>
<td>–</td>
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<tr>
<td>IT 40</td>
<td>49</td>
<td>2–3</td>
<td>++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3/15 455 days (14.17), TB:11/14 386 days (14.47)</td>
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times observed in Tgbov XV mice, the infectivity titres in the examined neuronal tissues was at a level of $10^{3}$–$10^{4}$ LD50 g$^{-1}$. A precise determination of the infectivity titre would require a complete end-point titration in Tgbov XV mice. The analysed tissues are in direct anatomical and functional connection to the brainstem; the optical and facial nerves represent cranial nerves (II and VII) and therefore have their origin in the brainstem. Also, the investigated sympathetic ganglia (ganglion cervicale craniale and ganglion cervicale caudale) are directly and indirectly linked to the brainstem and the spinal cord, singularly or in combination. Thus, an agent distributed along nerve fibres from the CNS to the samples under investigation can be easily explained.

In addition to the results from nervous tissues, we used a transgenic-mouse bioassay that revealed BSE infectivity in the tongue and nasal mucosa of clinically BSE-affected cattle, although no PrPSc deposition was detectable using highly sensitive methods. Due to the absence of PrPSc-specific labelling in the IHC analysis of these samples, it is impossible to define the cellular location of BSE involvement in these tissues. It can be assumed that the presence of infectivity in these tissues is a result of the radial spread from the CNS along small nerve fibres. This has already been described for other species such as hamsters (Thomzig et al., 2004), sheep (Andreoletti et al., 2004), mice (Bosque et al., 2002), non-human primates (Herzog et al., 2005) and humans (Kovacs et al., 2004). The spread to these tissues is probably a result of the direct connection of both non-neuronal tissues to the brain through their innervating cranial nerves XII, (nervus hypoglossus for the tongue) and I (nervus olfactorius for the nasal mucosa) (Schummer et al., 1987). This spread will, however, only occur at a late stage of the incubation and at low levels, since infectivity was only detected in animals showing clinical signs of BSE infection and we were not able to confirm PrPSc deposition in any of the samples analysed. The presence of PrPSc in the tonsillar tissue of the tongue has been demonstrated (Wells et al., 2005), but to our knowledge this is the first description of an accumulation of infectivity at a site far away from tonsillar tissue in the tongues of cattle.

Moreover, the spike experiment described above excludes the possibility of decreased amplification efficiency of the PMCA protocol applied because of the possible presence of certain compounds that might negatively influence the efficiency of this assay (such as those from erythrocytes) being in larger quantities in this tissue compared with brain tissue (Saa et al., 2005).

While it is widely acknowledged that the infectivity-detection mouse bioassay is distinctly more sensitive than the two biochemical PrPSc detection methods routinely applied, SAF immunoblot and IHC (Buschmann & Groschup, 2005), we were interested in comparing these results to those from the recently described PMCA method. This comparison revealed a similar detection limit for the mouse bioassay and the PMCA method for serial dilutions of a BSE-brain pool. This indicates that the PMCA method might, in the future, become an alternative method for the highly sensitive and at the same time very fast detection of trace amounts of BSE PrPSc. Recent studies (Saa et al., 2006; Castilla et al., 2006) have even described the PMCA method as having superior sensitivity compared with the animal bioassay. However, the approach was based on scrapie-infected hamster-derived PrPSc material, a hamster brain homogenate, as well as seven consecutive rounds of PMCA. Amplification of BSE-derived PrPSc material might be less effective under the standardized conditions applied here. Moreover, the lesser amplification efficiency of the PMCA method as compared with the mouse bioassay that became apparent in the present study may be due to a reduced PrPSc extraction efficiency from tongue tissue, which contains mainly connective tissue and muscle fibres that are difficult to homogenize completely. This might be an explanation as to why the PMCA method failed to detect PrPSc accumulation in the samples that were BSE-positive in the transgenic-mouse bioassay; to avoid any false-positive signals or de novo generation of PrPSc aggregates, only three rounds of PMCA were performed in our experiment.

We consider the fact that three highly sensitive detection methods (SAF immunoblot, IHC and PMCA) failed to detect PrPSc deposition in tissues where infectivity was easily detected by transgenic-mouse bioassay to be another indication that these two parameters are not as closely related as is commonly assumed. This phenomenon has already been described by others for BSE-infected C57Bl mice (Lasmezas et al., 1997) as well as for a transgenic mouse model expressing a PrP mutation that is linked with Gerstmann–Sträussler–Scheinker syndrome (Barron et al., 2007; Piccardo et al., 2007). The same holds true for scrapie-infected cell cultures where high titres of infectivity could be separated from PrPSc by centrifugation (Sun et al., 2008).

In this manuscript we report for the first time that BSE infectivity can be accumulated in the tongue and nasal mucosa of cattle in the clinical phase of a BSE infection. This fact may pose a health risk for the consumer, since to date tongue muscular tissue is not listed as specified risk material and is regularly consumed. Although we were able to demonstrate the high sensitivity of our Tgbov XV-adapted PMCA protocol, we were unable to decipher any PrPSc accumulation in these tissues that, however, contained considerable amounts of BSE infectivity.

**METHODS**

**Bovine samples.** The first set of samples derived from a collection of tissues that were taken under TSE-sterile conditions from clinically BSE-affected field cases in the UK (within the framework of the EU-funded project FAIR-CT98-3651). From these samples we selected neuronal tissues and ganglia, together with samples of the tongue and nasal mucosa from two cases, for further analysis (Table 1). The
tongue sample was collected from the tongue body; a transverse section from the dorsum, posterior to the fossa lingualis, 2 cm in depth and including mucosa, submucosa and striated muscle. The nasal-mucosa sample was dissected from the underlying cartilage and therefore included lamina propria and nasal gland tissue.

The second set of samples were derived from experimentally BSE-infected cattle, i.e. from the German BSE-pathogenesis study where every animal had been orally challenged with 100 g of brainstem material containing 10^{6.1} LD50 g^{-1} as determined in Tgbov XV mice (Hoffmann et al., 2007). Fourteen challenged animals, plus one mock-infected animal, that had been sacrificed up to 49 months after inoculation were selected for this analysis (Table 2). The tongue sample was taken as a transverse section approximately 10 cm from the tip.

Since each of the assays applied requires a specific sample buffer, it was impossible to prepare one homogenate for all tests. Therefore, small amounts of the tissue samples were prepared in the appropriate buffer for each method.

Mouse bioassay using bovine-PrP transgenic mice. A mouse bioassay was performed using selected tissues from both groups of animals (Tables 1 and 2) by using the highly susceptible Tgbov XV mice which overexpress bovine PrPSc (Buschmann et al., 2000). Ten percent homogenates of the samples were inoculated into 15 mice per group (30 μl intracerebrally). This challenge experiment was performed in a new animal facility where no TSE-infected animals had ever been kept. Only new equipment was used for the housing and care of the mice. The animals were checked for their health status every other day. Animals showing at least two TSE-specific clinical symptoms such as hind limb paresis, abnormal tail tonus, behavioural changes and weight loss over several consecutive days were sacrificed and samples were taken for diagnostic evaluation. Incubation times were calculated as the time between the inoculation and the death of the animal. All animals from the experiment were tested for PrPSc accumulation in their brains and only mice with positive results were included in the calculation of incubation times.

Detection of PrPSc accumulation in the brains of challenged mice. PrPSc accumulation was visualized either by PK digestion of brain homogenates followed by a small-scale preparation of SAF and a subsequent Western blot, or by IHC staining as described earlier (Buschmann & Groschup, 2005).

SAF preparation and immunoblot determination of PrPSc accumulation in bovine samples. Due to the limited amount of sample material available, most bovine samples were subjected to a small-scale preparation of SAF, as mentioned above, for the mouse brains.

The tongue sections were the only tissues where sufficient quantities of sample material were available for an SAF immunoblot using 2 g starting material, as recommended by the O.I.E. (Anonymous, 2004) and as described in more detail previously (Buschmann et al., 2006). This method is, so far, the most sensitive method for the detection of even minor amounts of PrPSc in a tissue.

Immunohistochemical PrPSc detection. In order to maximize the possibility of detecting even individual positive cells in the tissue samples analysed, we applied a serial cutting technique. First, three sections of 3 μm were taken, the following six to eight sections were discarded before collecting another three sections. This procedure was repeated five times, which enabled us to examine five different levels of the tissue block, with a distance of up to 141 μm between the first and the last section. Given a mean diameter per cell layer of 20 μm, at least seven subsequent cell layers can be analysed by applying this cutting technique.

For IHC analysis of these sections, tissue samples were processed as previously described (Hardt et al., 2000), with some modifications (Hoffmann et al., 2007). When using mAb 6C2, the slides were autoclaved in citrate buffer during the pretreatment process. The primary mAbs 12F10 (Cayman Chemical) and 6C2 (Rigter et al., 2007) were applied at dilutions of 1:150 and 1:50 in goat serum and incubated for 2 h at room temperature. A highly sensitive detection system using HRP-linked secondary antibody (EnVision HRP; Dako Diagnostics) was used with a 30 min room temperature incubation. The slides were developed in DAB (diaminobenzidinetetrahydrochloride) (Fluka Neu Ulm) and counterstained with Mayer’s haematoxylin.

All sections were examined by light microscopy.

PMCA. The published PMCA protocol (Castilla et al., 2005a, b; Saa et al., 2005) was used, with modifications. Tgbov XV transgenic-mouse brains (Buschmann et al., 2000) were used as a PrPSc substrate for the PMCA reaction. Sacrificed animals were perfused with PBS (pH 7.0–7.3) containing 5 mM EDTA prior to the removal of the brain. The brain samples were homogenized at 10% w/v in PMCA conversion buffer [PBS (Dulbecco’s PBS; Sigma) containing 150 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, and a complete protease inhibitor cocktail (cat. no. 1836145; Roche)]. Homogenates were centrifuged at 1500 g for 30 s and the supernatant was frozen immediately at −70 °C.

Bovine brain tissue which was used as a template for the positive-control PMCA reaction was homogenized at 10% w/v in PMCA conversion buffer and stored at −20 °C. Serial dilutions were prepared in PMCA conversion buffer. Analyte tissue samples were homogenized at 10% w/v in PMCA conversion buffer. Ten microlitres of the homogenate was suspended in 90 μl conversion buffer and stored at −70 °C. The tubes were placed into a thin-walled adaptor and put on a microplate module of a horn sonicator (model 3000; Misonix). Samples were exposed to 144 cycles of sonication for 20 s at a potency of 190–200 W (level 7), followed by an incubation for 30 min. The reaction was carried out in a 35 °C incubator. After the first round of 144 cycles, the 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.

We performed a standard PMCA protocol, including three consecutive rounds of PMCA, using PrPSc seeds from a BSE-positive cattle brain pool BBP12/92 (Fig. 3a). This BSE-positive brain homogenate was diluted 1:1000 in Tgbov XV brain homogenate (lane 1) and subjected to a first PMCA round of 144 cycles, which induced a seed-derived PrPSc amplification (lane 2). Samples were diluted 1:10 in Tgbov XV brain homogenate and subjected to a second PMCA round of 144 cycles (lanes 3–4). Again, samples were diluted 1:10 in Tgbov XV brain homogenate for a third PMCA round of 144 cycles (lanes 5–6).

PMCA products were incubated with 75 μg PK ml^{-1} for 60 min at 55 °C. After stopping the reaction with 0.5 mM PMSF, the samples were incubated with an equal amount of PBS/sarcosyl (4% w/v) buffer for 30 min at 37 °C and subjected to a PTA precipitation. The precipitation was carried out by the addition of 0.3% (w/v) PTA for 1 h at 37 °C with continuous shaking. After centrifugation 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 were performed as described above for the mouse and cattle samples.

Spike experiments were carried out using BSE-positive brain homogenate (end dilution 10−3) from sample IT13 with and without the addition of 10 µl of 10% tongue homogenate (sample KT 31) suspended in 90 µl Tgbv substrate. The analysis was carried out in quadruplicate. As a non-amplified control (NA-Co), a PTA precipitated brain sample from IT13 was used.

**Sensitivity of the detection methods applied.** A sensitivity assay was established in order to be able to compare the analytical sensitivity of the PMCA method and the mouse bioassay using Tgbv XV mice with the established diagnostic detection methods. We therefore prepared tenfold dilutions from a BSE-brain pool and used aliquots of these dilutions for analysis in the different test systems. This brain pool had been used for an end-point titration in Tgbv XV mice as well as for the oral BSE challenge of cattle and served as the BSE template in the PMCA positive-control reaction.

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**REFERENCES**


