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Pathogenesis of classical and atypical BSE in cattle

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ABSTRACT

It is known from earlier studies that the pathogenesis of BSE in cattle differs considerably from the TSE pathogenesis in sheep, where the lymphoreticular system (LRS) is majorly involved in the transport and propagation of the agent. In cattle, the BSE agent has only been detected in the Peyer's patches of the distal ileum and in the tonsils, which have both been identified as the portal of entry for the agent after oral uptake. It was shown that as opposed to most other animal species, in cattle the BSE agent amplifies almost exclusively in the central and peripheral nervous system. However, there is growing evidence for a centrifugal spread from the central nervous system into the periphery at the late stage of the disease. Moreover, there are only very limited data available concerning the pathogenesis of both atypical BSE forms, H type and L type BSE, as compared to classical BSE. In this manuscript we summarize the most recent data that we generated on the classical BSE pathogenesis after an oral challenge study that was performed with 56 cattle. Preliminary results on the pathogenesis of both atypical BSE forms are also presented, based on an intracranial challenge of cattle with German isolates of both atypical BSE forms.

1. Introduction

Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathies (TSE) that causes fatal neurodegenerative diseases in different species. BSE in cattle was first observed in the United Kingdom (UK) in 1986 (Wells et al., 1987) and has since then affected more than 180,000 animals in the UK and elsewhere. The oral uptake of BSE contaminated feed, mainly through concentrates and milk replacers fed to calves during the first 6 months of age, has been identified as the source of this epidemic shortly after its occurrence (Wilesmith et al., 1988). It has been estimated that more than three million infected animals in the preclinical stage were slaughtered and consequently entered the human food chain in the United Kingdom and elsewhere (Donnelly et al., 2002). As a result, the transmission of BSE infectivity to man has eventually 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 (data from National Creutzfeldt–Jakob Disease Surveillance Unit (NCJDSU), Western General Hospital Edinburgh, Scotland: Worldwide vCJD statistics 2010). The risk of human exposure to BSE is currently minimized in the European Union by BSE rapid testing of all cattle over 30 months of age (countries that could demonstrate a certain level of surveillance were able to change this age limit to 48 months for healthy slaughtered cattle and for fallen stock and other risk animals since January 2009) and by the removal of specified risk materials (SRM) from slaughtered

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cattle. These materials include the head and spinal cord of cattle over 12 months of age, the backbone including the spinal ganglia of cattle over 30 months of age, as well as the tonsils and the complete intestine independent of the animal's age.

This manuscript gives an overview on the current knowledge of the BSE pathogenesis and agent distribution in cattle, mainly based on a pathogenesis study after an oral challenge with classical BSE, and on experimental intracranial challenges of cattle with both atypical BSE forms.

2. The pathogenesis of classical BSE in cattle

The first BSE pathogenesis study was performed in the 1990s in the United Kingdom (UK) (Wells et al., 1996) where 30 calves at three months of age were challenged orally with 100 g BSE positive cattle brain macerate. This study revealed a very restricted agent distribution in BSE infected cattle (Wells et al., 1998). This knowledge was achieved by western blot and immunohistochemical analysis of tissue samples from the challenged cattle, as well as by inoculation of selected tissue homogenates into conventional RIII and C57Bl mice (Wells et al., 1998). It was shown that after oral exposure, PrP^{Sc} is initially accumulated in the ileal Peyer's patches starting 6 months after the challenge (Terry et al., 2003), and in the palatine tonsils starting 10 months after the challenge (Wells et al., 2005). In another study, samples collected from the ileal Peyer's patches, the tonsils and the central nervous system of cattle that were culled between 20 and 33 months after the challenge were used for a mouse bioassay in bovine PrP transgenic mice. While infectivity was detectable in the tonsil and Peyer's patches samples from all time points, the brainstem and sciatic nerve only contained infectivity after 27 months post challenge. These mouse bioassays confirmed the conclusion that infectivity is restricted to the nervous system, the ileal Peyer's patches and the palatine tonsil in BSE infected cattle (Espinosa et al., 2007).

3. The BSE pathogenesis study at the Friedrich–Loeffler-Institut

At the Friedrich–Loeffler-Institut (FLI), a follow-up study was performed where 56 calves were orally challenged with 100 g of a high-titre BSE brain macerate (10^8 ID_{50} per administered dose) that was kindly supplied by the Veterinary Laboratories Agency in Weybridge, UK (Hoffmann et al., 2007). Animals were clinically monitored and blood, liquor cerebrospinalis, urine and faeces samples were collected regularly. Every four months, a group of animals were euthanized and sectioned under TSE sterile conditions. More than 1000 individual samples were collected per animal during the necropsy. These tissue samples were analysed either by biochemical or immunohistochemical methods or by mouse bioassay using highly sensitive bovine PrP transgenic mice (Tgbov XV mice) (Buschmann et al., 2000). A first analysis of these samples revealed that the spread of the BSE agent from the gut to the central nervous system (CNS) mainly follows the parasympathetic and sympathetic nerve fibres of the autonomous nervous systems (Hoffmann et al., 2007). Moreover, mouse bioassays using Tgbov XV mice revealed an essential restriction of BSE infectivity to the nervous system of bovines, while samples of the lymphoid (other than the Peyer's patches) and reproductive systems were devoid of BSE prions (Buschmann and Groschup, 2005). Interestingly, using Tgbov XV mice, a very low infectivity load was detected in a sample of the *Musculus semitendinosus* which was most likely caused by the presence of terminal nerve fibres of the sciatic nerve in this muscle sample, which was demonstrated to contain substantial amounts of infectivity in the same experiment (Buschmann and Groschup, 2005).

4. Involvement of other parts of the digestive tract than the ileum

We were interested to decipher whether the ileum was the only portal of entry of the BSE agent or whether it could also be detected in other areas of the digestive tract. For that reason, samples of the Peyer's patches from the ileum, the ileocaecal junction, and the colon of animals sacrificed between 4 and 24 months after the oral challenge at FLI (between two and four animals per group) were analysed by biochemical (approved BSE rapid tests and a highly sensitive Western blot protocol including a precipitation of the pathological prion protein with phosphotungstic acid, PTA) and immunohistochemical methods. It has to be mentioned that the samples for the two rapid tests, for the PTA western blot and for the mouse bioassay were taken from the same Peyer's patch, while the sample from the immunohistochemical analysis had to be taken from a different Peyer's patch. In order to maximize the sensitivity of the immunohistochemical (IHC) analysis, we applied a serial cutting technique where five different layers of each tissue block were analysed at a distance of approximately 24 μm. This means that a total depth of 150 μm per analysed tissue block were examined in order to increase the possibility to even detect individual cells showing a PrP^{Sc} accumulation in the sample (for details of the methodology see Hoffmann et al., 2011).

Our results clearly support earlier reports that had identified the ileal Peyer's patches as the localisation of the earliest and strongest PrP^{Sc} deposition. Due to our highly sensitive IHC protocol, we were even able to visualize a PrP^{Sc} deposition in this area as early as 4 months after challenge. Moreover, it could be revealed that there is also a substantial PrP^{Sc} accumulation in the ileocaecal junction (starting from 12 months as compared to 4 months in the ileum), as demonstrated by biochemical methods and by IHC (Table 1). In contrast, no clear signals of a PrP^{Sc} deposition could be revealed for the jejunal samples (Hoffmann et al., 2011).

On a cellular basis, it could be demonstrated that the tingible body macrophages (TBM) display the earliest PrP^{Sc} accumulation. An immunostaining of follicular dendritic cells can firstly be seen 12 m.p.i, followed four months later, by cells of the enteric nervous system (ENS).

Mouse bioassays in Tgbov XV mice underlined the biochemical and IHC results in principle (Table 1). Infectivity was easily detected in the ileum samples collected between 8 and 20 months post infection (m.p.i.). Samples from earlier time points (1 and 4 m.p.i.) were not included in the

Table 1
Detection of PrP^{Sc} accumulation (IDEXX, PTA, IHC) and infectivity (bioassay) in the digestive tract samples (according to Hoffmann et al., 2011).

m.p.i.	Catte ID	Jejunum				Ileum				Ileocaecal junction			
		IDEXX	PTA	IHC	Bioassay	IDEXX	PTA	IHC	Bioassay	IDEXX	PTA	IHC	Bioassay
4	IT 19	neg	neg	neg	n.d.	neg	neg	pos	n.d.	neg	neg	neg	n.d.
	IT 45	neg	neg	neg	n.d.	neg	neg	pos	n.d.	inconcl	neg	neg	n.d.
8	IT 14	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg
	IT 20	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	neg
	IT 39	inconcl	inconcl	neg	pos	inconcl	pos	pos	pos	neg	neg	neg	neg
	IT 55	neg	neg	neg	pos	neg	neg	pos	pos	neg	neg	neg	pos
12	IT 01	neg	neg	neg	pos	pos	pos	pos	pos	inconcl	pos	neg	pos
	IT 16	neg	neg	neg	neg	inconcl	inconcl	pos	pos	neg	inconcl	neg	pos
	IT 06	neg	neg	neg	pos	inconcl	pos	pos	pos	neg	inconcl	pos	pos
	IT 57	neg	neg	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos
16	IT 07	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
	IT 28	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	pos	neg
	IT 46	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
	IT 65	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg	neg	pos
20	IT 10	neg	neg	neg	pos	neg	neg	neg	pos	inconcl	neg	neg	neg
	IT 17	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos	pos	pos
	IT 50	neg	neg	neg	neg	neg	neg	pos	pos	inconcl	neg	neg	neg
	IT 60	neg	neg	neg	pos	neg	neg	pos	pos	neg	neg	neg	neg
24	IT 26	neg	neg	neg	n.d.	neg	neg	pos	n.d.	neg	neg	neg	n.d.
	IT 24	neg	neg	neg	n.d.	neg	neg	pos	n.d.	neg	neg	neg	n.d.
	IT 47	neg	inconcl	neg	n.d.	neg	neg	pos	n.d.	neg	neg	neg	n.d.
	IT 58	neg	neg	neg	n.d.	neg	neg	neg	n.d.	neg	neg	pos	n.d.

m.p.i.: months post infection, IDEXX: IDEXX HerdChek BSE EIA, one of the approved BSE rapid tests, PTA: precipitation of the pathological PrP by phosphotungstic acid (PTA) followed by Western blot, pos: positive, neg: negative, inconcl.: a definite result (positive or negative) could not be determined, n.d.: not done.

bioassay. Infectivity was already detected at 8 m.p.i., and infectivity was also clearly present in the jejunal samples of animals sacrificed between 8 and 20 m.p.i., although at lower levels as compared to the ileum and ileocaecal junction (Hoffmann et al., 2011). These results clearly demonstrate the involvement of at least the ileocaecal junction and the jejunum besides the ileum in the early pathogenesis of BSE after oral infection. As a conclusion, the SRM definition as it is implemented in the EU can be considered satisfactorily safe, since the complete intestine of cattle, independent of the age, has to be destroyed. On the other hand, the restriction of the SRM removal to the ileum, as it is currently practised in North America, might leave the possibility for the BSE agent to enter the human food and animal feed chain.

5. The neuronal pathway—the four alternative ways to reach the brain

A neural spread after oral uptake of the TSE agent from the digestive tract via the enteric and sympathetic nervous system to the spinal cord was first suggested after an intragastric scrapie challenge of mice (Kimberlin and Walker, 1989). Afterwards, the neuronal pathway after oral uptake from the digestive tract to the brain has been analysed in detail using the scrapie 263 K hamster model (Baldauf et al., 1997; Beekes et al., 1998; McBride and Beekes, 1999; McBride et al., 2001). It could be demonstrated that the agent follows the vegetative projections of the sympathetic (N. splanchnicus) and parasympathetic (N. vagus) systems. In detail, splanchnic and vagus efferent (motor) and afferent (sensory) nerve fibres are followed either to the thoracic spinal cord (splanchnic nerves) or to the solitary tract nucleus and dorsal motor nucleus of the vagus nerve (Fig. 1). From the thoracal spinal cord, a centripetal and centrifugal spread to the cervical and lumbal spinal cord can be observed (McBride et al., 2001).

The oral BSE pathogenesis study performed with 56 cattle at the FLI represented a unique possibility to follow the neuronal transport of the BSE agent from the digestive tract to the brain during the different preclinical and clinical stages of the disease. We therefore analysed samples of the vegetative nervous system (sympathetic and parasympathetic) as well as the central nervous system (spinal cord) from animals that were sacrificed between 16 and 32 months after the oral BSE challenge using PTA-western blot, IHC and mouse bioassay in Tgbov XV mice. Preliminary results indicate that using the highly sensitive transgenic mouse bioassay, infectivity can be detected in parts of the sympathetic and parasympathetic system already at early timepoints in the incubation period. These findings are going to be presented in detail in a separate manuscript. Therefore the results of this study principally confirm for BSE infected cattle the same neurological pathways that have been described for scrapie infected hamsters.

6. Peripheral spread of the agent at the final stage of disease–following the same neuronal pathways as during pathogenesis

It has been shown by mouse bioassay in Tgbov XV mice that during the clinical phase of a BSE infection in cattle, a centrifugal spread of infectivity along nerve fibres into the periphery occurs (Buschmann and Groschup, 2005). As

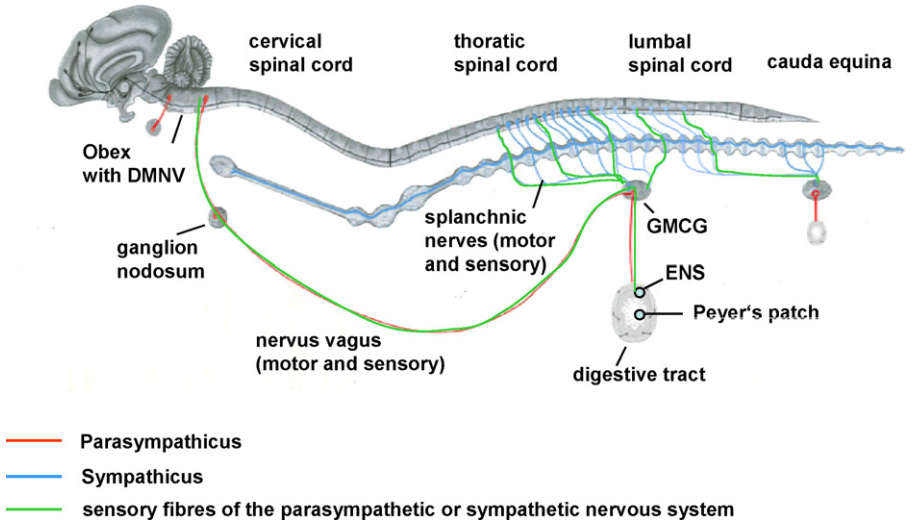

Fig. 1. Schematic diagram of the neurological pathway of the scrapie agent from the gastro-intestinal tract to the central nervous system of Syrian Gold Hamsters. After oral uptake, PrP^{Sc} is first detectable in the Peyer's patches and the enteric nervous system (ENS), followed by the ganglion mesenterium craniale/ganglion coeliacum complex (GMCG). From there, the agent may follow afferent (sensory) or efferent (motor) fibres of the parasympathetic and/or sympathetic nervous system. The parasympathetic pathway follows the nervus vagus to the ganglion nodosum and further to the dorsal motor nucleus of the vagus (DMNV), located in the obex. The sympathetic pathway on the other hand, follows the nervi splanchnici to the thoracic spinal cord and from there spreads into both directions (towards the brain and towards the cauda equina).

an example, the dorsal root ganglia (DRG) harbouring the neurons of the afferent (sensory) spinal nerves had been assumed to play a role in the BSE pathogenesis and agent propagation. However, several studies analysing samples from the British or the German BSE pathogenesis study came to the conclusion that the DRG are not primarily involved in the BSE pathogenesis, but are only secondarily infected by a centrifugal spread from the spinal cord into the DRG (Arnold et al., 2007; Hoffmann et al., 2007; Masujin et al., 2007). The finding that DRG may accumulate infectivity, as well as the fact that minor amounts of BSE infectivity were identified in a sample of the *M. semitendinosus* of a BSE field case in the final stage of the disease (Buschmann and Groschup, 2005), are of relevance for the protection of the consumer from an accidental exposure to the BSE agent. It was assumed that infectivity had been transported to the muscular tissue via the large sciatic nerve from the spinal cord at the final stage of the disease. These findings however argue for the systematic testing of slaughtered cattle above a defined age limit in order to certify the BSE negative status of tissues used for human consumption.

We were then interested to which extent such a centrifugal spread occurred in the late stages of the disease. Using the transgenic mouse bioassay, we were able to detect substantial amounts of infectivity in the tongue and nasal mucosa of clinically BSE affected cattle. Samples were collected from two British field BSE cases as well as six animals from the German BSE pathogenesis study that were sacrificed between 32 and 49 months after the challenge and that had all shown clear BSE-associated clinical signs (Balkema-Buschmann et al., 2011a). Interestingly, neither the highly sensitive SAF-immunoblot nor the recently published protein misfolding cyclic amplification (PMCA) method were able to reveal any PrP^{Sc} deposition in these samples (Balkema-Buschmann et al., 2011a). These results show that during the clinical stage of the disease, a centrifugal spread of infectivity does occur regularly into tissues that are in direct contact with the CNS via cranial nerves or via large peripheral nerves. This fact has to be taken into account when considering modifications of the consumer protection measures.

7. Pathogenesis and agent distribution in cattle experimentally challenged with atypical BSE

After the detection of two so far unknown forms of atypical BSE in 2004 (Biacabe et al., 2004; Casalone et al., 2004), the question of the zoonotic potential of these TSE forms needed to be answered. Both forms have so far only occurred in catttle that were at least eight years old. It has therefore been postulated that they represent a spontaneous TSE in cattle, comparable to the majority of sporadic Creutzfeldt–Jacob disease (CJD) cases in humans (Will et al., 1998). While in L type BSE, the molecular mass of the lowest protein fragment representing the unglycosylated form of PrP migrates slightly lower than that in classical BSE (Casalone et al., 2004), in H type BSE the same band migrates a little bit higher as compared to classical BSE (Biacabe et al., 2004). In the initial description of L type BSE, it had already been reported that the distribution of PrP^{Sc} in the different brain regions did not exactly follow the pattern that was known for classical BSE. This finding may question the possibility to detect such cases, as in weak cases of L-type BSE the obex and brainstem regions, representing the target areas for all BSE diagnostic methods, may not give positive results in the diagnostic tests. In the brains of the first cow where this BSE form was detected, the brainstem and obex area did not display the highest PrP^{Sc} concentrations, but instead, abundant amounts were detected in the thalamus and

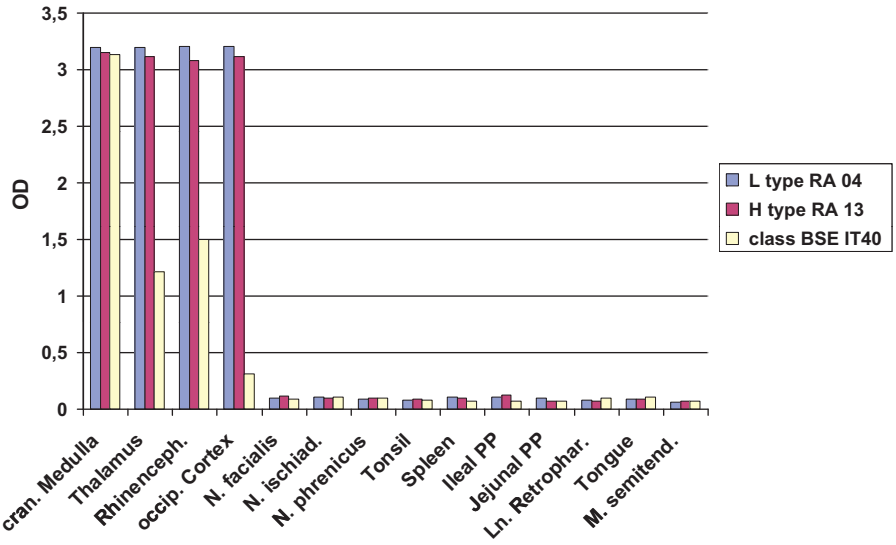

Fig. 2. PrP^{Sc} distribution in peripheral tissues of cattle clinically affected with classical BSE, H type BSE or L type BSE. Samples were collected under TSE sterile conditions with single use instruments. Analysis was performed using the IDEXX HerdChek BSE EIA, which is one of the BSE rapid tests that have been approved by the EU authorities for the testing of bovine obex samples.

olfactory region (Casalone et al., 2004). Moreover, transmission experiments to human (Kong et al., 2008) and bovine (Buschmann et al., 2006) transgenic mice as well as macaques (Comoy et al., 2008) had revealed clear indications for a higher zoonotic potential of this BSE form than classical BSE.

We were therefore interested to reveal the agent distribution in cattle incubating or already clinically affected with both atypical BSE forms. For that reason we performed a challenge experiment in cattle that, due to the very limited amount of inoculum, had to be done intracranially. Five and six animals of the Holstein-Friesian breed were challenged with 1 ml of a 10% brainstem homogenate of a German isolate of the H-type or the L-type BSE form, respectively. At selected time points after the inoculation, individual animals were scheduled to be euthanized and sectioned under TSE sterile conditions. However, since the incubation time turned out to be shorter than anticipated, the majority of the animals developed clinical signs of a BSE infection between 12 and 16 months after the challenge and had to be euthanized immediately for animal welfare reasons. Only one animal challenged with L type BSE was negative in all tests, while the remaining 10 animals were highly positive when the brainstem sample was tested in an approved BSE rapid test. The detailed experimental design and progress of this experiment is described elsewhere (Balkema-Buschmann et al., 2011b). A pilot study using samples derived from one animal each challenged with classical, H type and L type BSE, collected from different brain regions and different peripheral tissues from the lymphoreticular system, the peripheral nervous system and the muscular system was performed using one of the approved BSE rapid tests. According to this study that is based on an intracranial inoculation of the agent, the modified PrP^{Sc} distribution pattern in the brain could be confirmed for both atypical BSE forms. However, the PrP^{Sc} distribution in the analysed peripheral tissues seems to follow the pattern that is known for classical BSE, meaning that no relevant PrP^{Sc} depositions were detectable in any of the analysed peripheral tissues (Fig. 2). This result has an important impact on the consumer protection, as it can be assumed that despite the higher zoonotic potential of the L type BSE form, there is not an elevated risk for the consumer as long as BSE rapid tests are applied to all slaughtered cattle above a certain age limit.

8. Conclusions

In this manuscript we summarize the actual knowledge of the BSE pathogenesis in cattle. These findings are crucial for the definition of the most effective and at the same time the most practical measures for the prevention of a re-circulation of the agent from one of the BSE forms in the human food or in the animal feed chain.

Conflict of Interest

None.

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