CHAPTER ELEVEN

Phenotypical Variability in Bovine Spongiform Encephalopathy: Epidemiology, Pathogenesis, and Diagnosis of Classical and Atypical Forms

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Abstract

After thirty years, bovine spongiform encephalopathy (BSE) still represents the biggest crisis in the field of food safety. Initially detected in the United Kingdom in 1986, BSE spread to many other countries all over the world, involving approximately 200,000 cattle. The origin of BSE is uncertain, but epidemiological studies suggest that the source was cattle feed prepared from prion-infected animal tissues. The implementation of the drastic measures, including the ban of meat and bone meal from livestock feed and the

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removal of specified risk material from the food chain, has eventually resulted in a significant decline of the epidemic. For many years, it was believed that the disease was caused by transmission of a single prion strain. However, since 2004 two types of BSE, with distinct phenotypical characteristics, have been detected in Italy and France. These atypical types are characterized by distinct Western Blot profiles of abnormal protease-resistant prion protein, named high-type (H-BSE) and low-type (L-BSE). At present, there is no comprehensive information about the origin of the atypical BSEs (sporadic vs. acquired), and data about the pathogenesis of both atypical forms are very limited as compared to the classical type (C-BSE). This chapter will provide a well-organized overview of what is known about classical and atypical BSE. It will review information on the main epidemiological features, pathogenesis, and the criteria for routine diagnosis based on rapid tests, histological, immunohistochemical, and Western blot analysis. Furthermore, a brief overview about the most recently in vitro techniques will be also provided.

1. INTRODUCTION

Bovine spongiform encephalopathy (BSE), first diagnosed in the United Kingdom (UK) in 1986, is now classified as a potentially lethal zoonotic disease acquired *via* contaminated food. BSE spread from the UK to at least 28 other countries, mostly in Europe, with occasional cases also confirmed in Asia (Japan), the Middle East (Israel), and North America. To date, more than 112.000.000 animals have been examined in Europe, and more than 184.500 cases of BSE have been confirmed in the UK, 5.500 in Europe, and 60 in the rest of the world (Brazil, Canada, Israel, and Japan). The World Organization for Animal Health (OIE) reported only 5 cases of BSE worldwide in 2015, 3 of which for Europe, 1 for Norway, and 1 for Canada; in 2016 only 2 cases, in France and in Spain and in the current year, only one BSE case has been reported for Ireland.

While the origin of BSE remains unknown, the cause of its spread is well recognized. In fact, the use of ruminant-derived meat and bone meal (MBM) as a food source for animals is the most reliable hypothesis of its diffusion in cattle populations. MBM contains central nervous system (CNS) tissues and other tissues that are known to contain the protease-resistant, disease-associated isoform of the prion protein (PrPSc).

BSE has a long incubation period of about 2.5 to 8 years, with clinical disease usually affecting adult cattle at a peak age onset of 4 to 5 years, and all breeds being equally susceptible. BSE is characterized by altered behavior, abnormal posture changes, incoordination and difficulty in rising, decrease in milk production, and loss of body weight. Histopathologic

features include neuronal and neuropil vacuolation, glial reaction, and the complete absence of inflammatory lesions.

In addition to the classical form of BSE (C-BSE), two atypical BSE prions were identified in France and in Italy in 2004.[1,2] One has higher molecular mass fragments than C-BSE and is called H-BSE; the other has a lower molecular mass and is called L-BSE, or bovine amyloidotic spongiform encephalopathy (BASE), because of the presence of PrP-positive amyloid plaques in the brain. The most likely hypothesis is that they arise spontaneously in cattle, comparable to most sporadic Creutzfeldt-Jakob diseases in humans. From an epidemiological point of view, these atypical forms are mainly detected in cattle 8 years of age or older. Most cases were identified in fallen stock and none was reported as clinically suspected, suggesting that the clinical presentation is unlike C-BSE. Since 2001, a total of 44 cases of L-type and 60 of H-type BSE have been identified in Europe according to the European Union BSE databases. The prevalence of atypical BSE cases in the rest of the world is unknown because official surveillance requirements or systematic reports are lacking.

2. EPIDEMIOLOGY

During the mid eighties routine surveillance activities carried out on the English livestock led to the identification of an emerging neurological disease affecting an increasing number of cows. In some ways the disease features were resembling scrapie, i.e., a known disease affecting small ruminants. The combination of the peculiar clinical presentation with the histopathological pattern observed at the brain level and the discovery of protein fibrils reminiscent scrapie-associated fibrils allowed the formulation of an intriguing hypothesis: a bovine spongiform encephalopathy was emerging and spreading among the cattle.[3] One year later the results from an inoculation experiment confirmed the nature of the disease[4] as a *transmissible spongiform encephalopathy*. The retrieval of archived bovine brains indicated that the first cases occurred around 1985. Based on data from nearly 200 affected herds and cases of BSE, the initial epidemic curve was consistent with that of an extended common source epidemic.[5] The emerging cases were geographically spread but there were no clear direct epidemiological relationships linking them. Most of the cases were from dairy herds of large size. All those data helped in formulating a feedborne hypothesis for the diffusion of such a spongiform disease in cattle. In particular, attention was focused on one ingredient: the inclusion in feed of infected meat and bone

meal might have accounted for the transmission to cattle. A subsequent case–control study[6] provided evidence of a higher risk of disease in dairy cows herds where concentrated proprietary feed had been massively used. The hypothesis is still largely accepted and international supportive evidence has been cumulated over the years.[7–10]

To understand why meal and bone meal might have represented a new hazard, a survey of all rendering plants in operation in the UK was carried out in 1988.[11] Two major changes in the industrial processes were identified as possibly significant, i.e., a change from batch processing to continuous processing and a reduction in the use of hydrocarbon solvents to maximize the extraction of tallow: it was hypothesized that whereas the original processes through a combination of time and temperature had been able to inactivate the agent responsible of BSE, the recent changes were no longer able in preventing the infections in orally exposed cattle. Moreover the survey also revealed a geographical variation in the use of hydrocarbon solvents and in heat treatment which was negatively correlated with the incidence of BSE, as the process was abandoned entirely in England and Wales, but remained in use in Scotland where BSE was showing the lowest incidence. This finding provided the first explanation for the variation in the geographical distribution of the BSE risk.

Based on the feedborne hypothesis, an early ban (1988) of the administration of MBM to ruminants was the first effective measure applied in the UK: consistently, after peaking in 1992 (with more of 37,000 confirmed cases) the epidemic started to decline. Moreover, to further reduce the risk in 1989, a new measure was enforced, i.e., the removal of all offal from both the food and feed chains.

The spread of the disease in other European countries (initially Portugal, Ireland, and Switzerland)[12] and above all the discovery of a pathogenic relationship between BSE and a new fatal neurodegenerative disorder in humans, called variant Creutzfeldt-Jakob disease (vCJD), prompted the European Commission to commit an EU-wide risk assessment exercise to quantify the risks that Member States were facing.

In particular, the concern on the human risk of exposure to BSE *via* contaminated food was suggested after the identification in the UK of the first few cases of vCJD.[13] Besides the epidemiological observation of the emerging of a new human disease in a country that was experiencing the largest livestock epidemic, molecular and biological studies of strain-typing provided evidence that the human and animal diseases were sharing the same agent.[14–17] BSE was recognized as a zoonotic disease that was posing a

serious risk to human and animal health. Risk assessment was identified as the most appropriate tool to address the urgency for the European Community to devise effective interventions aimed at the protection of public health through specific rules for the prevention, control, and eradication in cattle.

2.1 Geographical Risk Assessment

One of the main reason to seek help from risk assessment was the difficulty in obtaining a reliable epidemiological situation through routine surveillance activities: the disease was relatively rare, difficult to diagnose (only post mortem), and the farmers were not willing to report as each case was representing a stigma. In the first stages of the epidemic, the passive surveillance, based on a voluntary reporting of the cases, was the only detection strategy and resulted in a biased assessment of the spread of the disease[18] and of its risk factors. An enforced active surveillance system was made available in the majority of the European Countries only since 2001: it helped in facing the lack of an accurate data collection and in understanding better the risk factors.

Meanwhile in the second half of nineties, through its Scientific Steering Committee (SSC), the European Commission decided to designate Transmissible spongiform encephalopathies (TSE) working group formed by international experts for developing and applying the risk assessment method in establishing the risk that a country could hold undetected BSE cases in cattle population.[19]

The result was a risk assessment process entitled the Geographical Bovine Spongiform Encephalopathy Risk (GBR). The main outcome of the GBR methodology was to devise a model enabling the classification of the risk of a country to have at least one animal being infected with the BSE agent in its autochthonous bovine population. Where its presence had been already confirmed, the GBR was able to provide an indication of the level of infection and spread.[20] The GBR is based on a general model aimed to assess the likelihood of the introduction of the BSE agent in a country, its potentially recycling and amplifying or elimination.[21] It takes into account and embodies in a conceptual frame the main risk factors involved in determining the disease: the main assumption is that BSE could be initially introduced through the importation of contaminated MBM or live cattle from BSE-risk countries (the so-called external challenge); the BSE agent could potentially enter into the rendering system, and the rendering system itself could allow the amplification of the BSE agent in processing animal by-products. A country may be considered a stable system if it does not allow the BSE

agent to propagate following its introduction and it is capable to prevent the import of or remove BSE infected animals before processing.

The GBR was a useful tool to provide a general frame for the epidemiology of the disease and to assess the BSE status of a country in order to support the European Commission in establishing legislation rules for international trade of cattle and their products.

2.2 Risk Factors and Effective Measures

As mentioned, in general, dairy cattle have a higher risk than beef suckler herds because of the greater use of compound feed.[6,10] Herd size has been associated to the spread of infection, even though it was not consistently across countries.[10,22–24] After the 1992 peak, the rate of decline in epidemic in the UK was not as important as expected. To identify the drivers of such a persistence of the disease within the population ad hoc studies were carried out: such studies, looking at animals from the birth cohorts before and one year after the July 1988 when the initial feed ban on feeding ruminant-derived MBM to ruminants entered into force, demonstrated the role of low-level cross-contamination of cattle feed by pig feed in BSE incidence as the epidemic evolved.[25,26] Finally, there is no evidence of animal to animal spread, but a cohort study indicated that a very low level of maternal transmission, insufficient to maintain the epidemic, may have occurred in the UK.[27]

The international legislation to effectively manage the risks associated with BSE has been devised on the basis of the identification of the mentioned risk factors. The European Regulation (EC) 999/2001 is an integrated legislative rule to manage the BSE crisis and to prevent, control, and eradicate the disease. It entered into force in 2001 and provided a strict harmonized set of rules to be implemented in all Member States. The regulation aimed at avoiding the exposure of the cattle population through potentially infected meat and bone meal also due to cross contaminations at feed mills or at farms: for the purpose an extended total feed ban preventing the administration of feed containing mammalian-derived protein to all farmed animals was enforced; the measure was combined with the removal of specified risk material from all slaughtered animals (bovines and small ruminants) to avoid the amplification of the BSE agent through the rendering process. Other prevention and control measures contained in the regulation are surveillance measures aimed to detect and eliminate BSE-infected animals.

Over time the trend of the C-BSE and, thus, the effectiveness of enforcement of the measures were monitored through the testing of a huge number

of cattle (10 million cows per year on average in EU). The surveillance made clear that BSE occurred in European countries and throughout the world with more than 190,000 BSE cases in 28 countries worldwide. Most reported cases are from the UK; in other countries the epidemic peaked ten years later, in 2002 or 2003. In particular, in the UK, more than 184,000 cases of BSE have been reported and more than 3 million cows were destroyed to stop the spread of the disease. Then, following the ban on feeding ruminant-derived MBM and the total feed ban, the occurrence of BSE was dramatically reduced.

After many years, the BSE problem seems to be solved now, and since 2005 the European Union has been setting up an exit strategy from the crisis,[28] providing for the gradual easing of the measures. Today, a drastic reduction in the extent of surveillance has been enforced in EU, resulting in the restriction of the testing in most of the European Member States to the so-called at-risk groups (fallen stock and emergency slaughtered cattle): such a residual surveillance is of outmost importance as it represents an early warning tool in the case of an eventual re-emerging of the disease.

3. PATHOGENESIS OF CLASSICAL AND ATYPICAL BSE

Because the epidemiological and clinical characteristics of C-BSE make it difficult to study its pathogenesis in field conditions, the bulk of pathogenetic data are derived from experimental transmission studies. A number of knowledge gaps remains, however. How the prion agent crosses the epithelium after oral exposure to infective material is not fully understood. The most likely mechanism is *via* M-cells, which are highly phagocytic epithelial cells found within the epithelium that covers Peyer's patches (PP) and specialized in the transport of large particles and whole bacteria across the gut epithelium.[29] In prion diseases, these cells are able to transcytose the prion protein from the lumen of the gut into the epithelium. During the first 8 months post-infection (mpi), the earliest PrPSc accumulation is displayed by tingible body macrophages (TBM). TBM belong to a subset of mononuclear phagocytes found in the unique microenvironment of the germinal centers in gut-associated lymphoid tissue (GALT) of the ileocecal junction and jejunum and in the PP of the ileum.[30]

Infectivity can be found in the palatine tonsils at 6–10 mpi.[31] A peak of infectivity in the distal ileum at 12 mpi is related to the number of follicles involved and to the amount of PrPSc detectable in the follicular dendritic cells (FDC) and TBM, indicating increased clearance activity of these cells.

FDC are stromal cells residing in primary follicles and in the germinal centers of secondary and tertiary lymphoid organs. There is a second peak of PrPSc accumulation at 24 mpi, where prion protein is mainly found in the TBM and FDC of the jejunum and ileum. A third peak of PrPSc accumulation may be present, with some individual variability, between 32 and 40 mpi in the ileal PP.[32]

The enteric nervous system, and the involvement of nerve fibers of the mucosal plexus in particular, represents the way of entry of the prion into the nervous system.[33] Through the mesenteric nerves, the prion proteins accumulate in the cranial celiacomesenteric ganglion complex and then ascend to the thoracic spinal cord *via* the sympathetic nervous system (e.g., splanchnic nerves) and to the brainstem and the brain *via* the parasympathetic nervous system (e.g., vagus nerve) and nodose ganglion.

From the thoracic spinal cord, PrPSc spreads rostrally to the cranial medulla and caudally to the cauda equina.[34] From the spinal cord, PrPSc then accumulates in the dorsal root ganglia, trigeminal ganglia, and cervical ganglia.[35] The adrenal glands and sciatic nerve have also been described as positive tissues with demonstrable prion protein accumulation.[36] Between 42 and 84 mpi, PrPSc can be identified in the spindles of various muscles, including the masseter, triceps brachii, intercostal muscles, and semitendinosus.[37]

Comprehensive information about the pathogenesis of atypical BSEs in cattle is lacking. Some data are available on the peripheral distribution of H- and L-BSE agents in cattle experimentally challenged *via* the intracerebral route, but all the animals in these studies were allowed to develop clinical disease. Experimental transmission studies have demonstrated that in both H-BSE and L-BSE PrPSc accumulates in CNS tissues, peripheral ganglia and nerves, muscles (muscle spindles), adrenal glands, and retina.[38] No lymphoid tissues or gastrointestinal tissues have tested positive in atypical cases. Furthermore, a study of intraspecies transmission of a case of L-type BSE suggested that prions may propagate in the CNS and spread centrifugally by nerve pathways.[39]

4. SAMPLING

When clinical suspected cases are under investigation, the whole brain is sampled in order to go through a classical neuropathological approach to examine a representative number of cerebral areas. To allow a reliable BSE diagnosis, at least one block 0.5–1 cm in width taken from the medulla oblongata at the level of the obex[40] must be subjected to histological and

immunohistochemical examination, respectively, for spongiform changes and PrPSc detection, performed on formalin fixed and paraffin-embedded tissue; furthermore immunoblot for PrPSc will be carried out on a complete coronal section taken immediately rostral or caudal to the above area.

The remaining portion of the brain tissue will be placed in 10% buffered formalin to be subjected to further neuropathological examination in case of negative results for BSE.

For monitoring purposes through active surveillance, rapid tests require a precise sampling within 1.0 cm rostral or caudal to the obex, in order to include key target sites of PrPSc accumulation. For this purpose brainstem is collected with the help of a dedicated spoon through the *foramen magnum* (Figs. 1 and 2). Sampling for rapid testing should be carried out

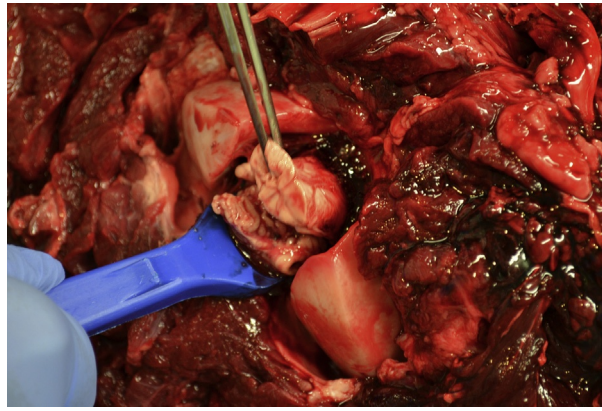

Fig. 1 Sampling of brainstem through the foramen magnum.

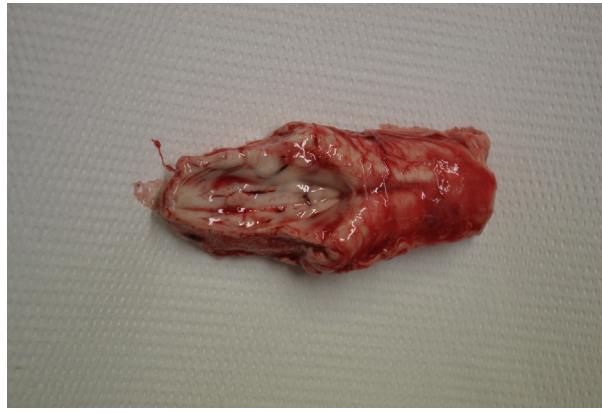

Fig. 2 Fresh brainstem at the level of the obex.

from a hemi-section of the medulla in order to preserve the contralateral one for further confirmatory tests and a possible discriminatory Western Blot (WB).

In case of autolysis of the sample, an unidentified aliquot of the medulla should be taken and tested. When a positive result is achieved, this has to be considered a valid result, while a negative test cannot be considered as a negative animal and has to be reported as appropriate (i.e., negative result in not suitable tissue).

5. RAPID TESTS

Rapid testing for BSE monitoring purposes was first introduced in the European Union in January 2001 to strengthen control programs on slaughtered animals and fallen stock. The systematic sampling of animals post-mortem in order to verify the impact of control measures and to allow the trend of the disease to be followed allowed the detection of the first BSE case in most European countries as well as of the majority of BSE cases.

Rapid tests became available for BSE active surveillance in the late 1990s and their suitability for this purpose was assessed through three evaluation exercises implemented since 1999. While in the first comparative study only the accuracy and the analytical sensitivity of the rapid tests under evaluation were assessed, robustness on poor quality negative samples and a field trial were later included in order to verify their suitability for routine activity. In 2009 the Community Reference Laboratory for TSEs assessed the analytical sensitivity of all the currently approved TSE rapid tests to determine their continued suitability for active surveillance plans.[41] Importantly, the design strategy of this study involved all tests being evaluated against the same sample set. The lowest limit of detection (LOD) of rapid tests approved for the diagnosis of classical BSE in bovines was assessed as a crucial parameter for their suitability. According to EFSA requirements for the evaluation of TSE rapid *post-mortem* tests[42] a new test will be approved for full evaluation if the LOD is better than, similar to, or no more than 2 logs poorer than the most sensitive test. This last point is considered to be a critical point for the key issue of public health and for the capacity of a test to detect BSE in cattle at early stages of incubation.

To date European Commission (EC) has assessed 19 rapid tests, 9 of which were approved for survey including a new version of the Enfer TSE test for BSE diagnosis.[43]

All currently recognized forms of BSE are detectable by these methods although an official full sensitivity and specificity evaluation for atypical forms (H- and L-types) has not been carried out. However a recent study demonstrates the suitability of BSE EU-approved rapid tests also for the detection of both atypical forms.[44]

Tests approved for active surveillance activity within the EU are listed in Annex C Chapter X of the TSE Regulation 999/2001 and subsequent amendments. To date, according to the most recent Regulation 1148/2014, the following tests can be used:

- the immunoblotting test based on a Western blotting procedure for the detection of the Proteinase K-resistant fragment PrPRes (Prionics-Check Western test)
- the sandwich immunoassay for PrPRes detection (short assay protocol) carried out following denaturation and concentration steps (Bio-Rad TeSeE SAP rapid test)
- the microplate-based immunoassay (ELISA) which detects Proteinase K-resistant PrPRes with monoclonal antibodies (Prionics-Check LIA test),
- the two-sided immunoassay using two different monoclonal antibodies directed against two epitopes presented in a highly unfolded state of bovine PrPSc (Roboscreen Beta Prion BSE EIA Test Kit).
- the immunoassay using a chemical polymer for selective PrPSc capture and a monoclonal detection antibody directed against conserved regions of the PrP molecule (IDEXX HerdChek BSE Antigen Test Kit, EIA &HerdChek BSE-Scrapie Antigen (IDEXX Laboratories)).
- the lateral-flow immunoassay using two different monoclonal antibodies to detect Proteinase K-resistant PrP fractions (Prionics Check PrioSTRIP).

All these tests, apart from the IDEXX HerdChek tests, that are based on a conformational detection technology for PrPSc using a specific aggregate capture ligand on a dextran polymer (Seprion ligand technology, Microsens Biotechnologies, London, UK), rely on the digestion of the cellular prion protein (PrPC) to allow the binding of anti-PrP antibodies to the PK resistant PrP.

The Prionics-check Western blot was the first test available for BSE surveillance. It is based on an optimized WB procedure that allows to monitor the characteristic three band pattern (non-, mono-, and di-glycosylated forms) of the protein-resistant PrPSc fragment (27–30 kD) and their position relative to the control, the top band corresponding to a protein of approximate molecular weight of 30 kD.

The other tools are represented by a lateral flow immunoassay and four semiquantitative ELISA techniques that provide qualitative results relative to a cut-off value.

Such tests provide a preliminary diagnosis from which positive or inconclusive results are subject to examinations by histology, immunohistochemistry, or WB confirmatory methods.

Whenever their first purpose is that of screening methods, for which a positive or suspect result must be followed by a confirmatory method, a combination of two rapid tests is allowed for primary screening and subsequent confirmation, according to an algorithm maintained in the TSE-LAB-NET.[45] However, a negative result according to the two rapid tests as well as a discordant one has to be further investigated through a traditional confirmatory test. Where the histopathological examination is used for that purpose, but proves to be inconclusive or negative, the tissues must be submitted to a further examination by one of the other confirmatory methods and protocols.

6. HISTOPATHOLOGICAL EXAMINATION

Histological examination is performed on formalin-fixed, paraffin-embedded brain sections stained with hematoxylin and eosin. The solitary tract nucleus (NST) and the spinal tract nucleus of the trigeminal nerve (NSTV) are the target areas at the level of the obex to be examined in order to diagnose C-BSE (Figs. 3 and 4). Vacuolation of the gray matter

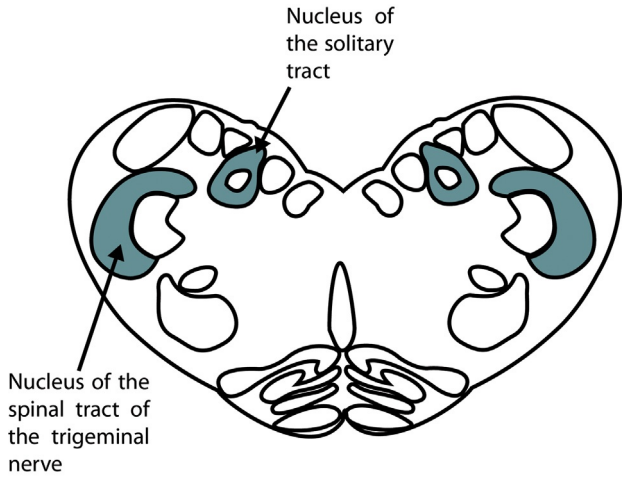

Fig. 3 Section of the obex showing the target nuclei for BSE diagnosis.

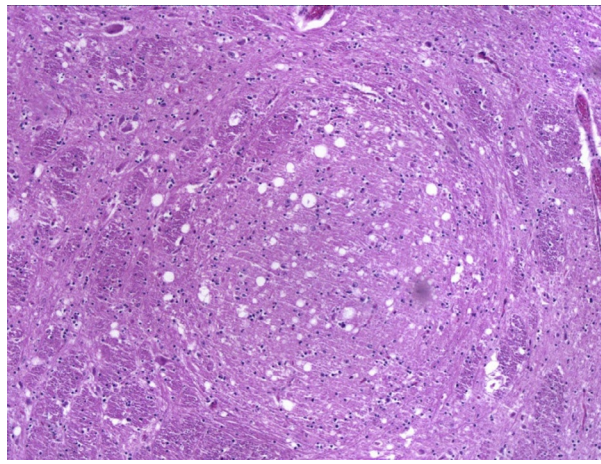

Fig. 4 H&E, nucleus of the solitary tract of C-BSE: presence of spongiosis in the neuropil (20 ×).

neuropil (spongiform change) and/or vacuolation of neurons, not necessarily associated with astrocytosis and neuronal degeneration, are the most frequent histological changes that can be observed in the CNS of C-BSE cases.[46–48] Neuronal vacuolation is especially prominent in the vestibular nucleus in the medulla oblongata at the level of the cerebellar peduncles. Other histological lesions are vacuolation in the central gray matter of the midbrain and mild spongiform changes of the neuropil at the level of the thalamus.

In natural cases of L-BSE (BASE), spongiosis is not consistently found in the brainstem, at the level of the obex or in more rostral areas. The frontal, parietal, and occipital cortices are apparently spared, and no vacuolation is detected in the olfactory bulb, piriform cortex, and hippocampus.[1] A more severe involvement of the central gray matter (periaqueductal gray) and rostral colliculus, but not the vestibular nuclear complex, has been observed in experimental cases of L-BSE. Additional brain areas, including the olfactory area, amygdala, hippocampus, and dorsal horns of spinal cord, are severely involved. The ventral and dorsal roots do not show major pathological changes.[49]

Vacuolar changes are generally observed in all the brain areas in experimental H-type BSE. Major vacuolation has been described in the thalamic nuclei and the neuropil of the central gray matter of the midbrain; however, mild vacuolation can also be present in the caudal cerebral and cerebellar cortices. Spongy changes in the vestibular and pontine nuclei are not as prominent as those seen in the other brainstem nuclei.[50]

7. IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis is performed on paraffin-embedded brain tissues to highlight the presence of PrPSc accumulation. The slides are dewaxed, rehydrated, and then immersed in 98% formic acid for 25 min. After washing in distilled water, the sections are autoclaved at 121°C. Endogenous peroxidase activity is blocked in 3% hydrogen peroxide. To block nonspecific tissue antigens, the sections are incubated with horse blocking serum and then incubated with a primary monoclonal antibody directed to prion protein. After rinsing, a biotinylated secondary antibody is applied to the tissue sections, followed by the avidin-biotin peroxidase complex. Prion protein immunoreactivity is visualized using 3,3-diaminobenzidine as chromogen; the sections are then counterstained with Meyer's hematoxylin.

Several morphologic types of PrPSc deposition can be observed in the brain of C-BSE affected cattle[51] (Fig. 5):

Glial type: Radiating, branching PrPSc deposits often centered on a visible glial-type nucleus, conferring a star-like appearance, are predominantly present in the central gray matter, the cerebral lamina, and within the medial pontine nuclei in the cerebral cortex, thalamus, and obex.

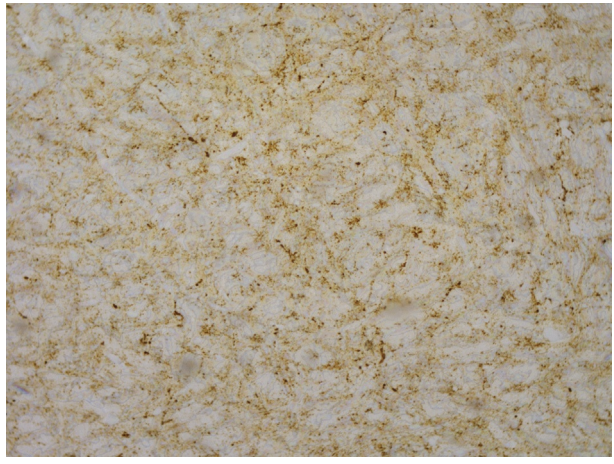

Fig. 5 IHC, patterns of PrPSc of C-BSE, characterized by granular deposits and linear tracts (10 ×).

Granular type: Numerous small PrPSc granules are commonly found in the neuropil of the gray matter nuclei, such as the dorsal motor nucleus of vagus nerve (DMV), NST, and in the thalamic nuclei.

Intraneuronal type: Fine to coarse, sometimes confluent, granular deposits of PrPSc in the neuronal cytoplasm are often observed in the DMV, reticular formation, olivary nuclei, vestibular complex, pontine and thalamic nuclei, and hypothalamus.

Perineuronal type: Thin deposits of PrPSc are observed around individual neuronal perikarya and neuritis in the caudate and putamen nuclei of the basal ganglia and in the DMV.

Linear type: Thread-like deposits of PrPSc are seen along neuronal processes, particularly at the level of the reticular formation of the brainstem.

Coalescing type: Large granular PrPSc deposits merging to form amorphous or mesh-like masses are noted.

Intraglial type: Fine punctate PrPSc is found adjacent to glial nuclei.

A distinctive feature of natural cases of BASE is the presence of PrPSc deposition prevalently in the more rostral portions of the brain than as occurs in C-BSE. PrP-positive amyloid plaques are the hallmark characteristic of BASE: they appear as dense, unicentric, or less frequently multicentric, round structures up to 25 μm in diameter with a pale core and a dark radial periphery (Fig. 6). They are predominantly located in the thalamus, subcortical white matter, the deeper layers of the cerebral cortexes, and the

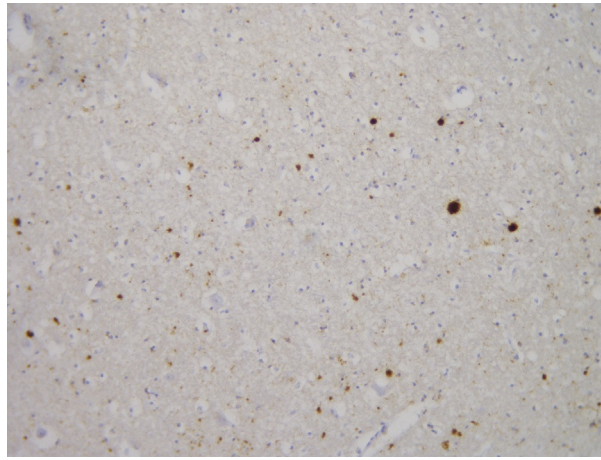

Fig. 6 IHC, patterns of PrPSc of L-type BSE (BASE), characterized by amyloid plaques (20 ×).

olfactory bulb. Other prevalent PrPSc deposition patterns are the punctate and granular types, which are mildly present in the hypoglossal and olivary nucleus and moderately present at the level of DMV nucleus, NST, NSTV, and reticular formation.[52] Glial, intraneuronal, perineuronal, and linear tracts are also frequently observed in different brain areas in BASE cases.

In experimental BASE cattle, abundant amyloid PrP-plaques are found in the subcortical white matter and deep gray nuclei, as observed in natural BASE cases. No PrP-plaques are present in the olfactory glomeruli, the cerebellum, or the spinal cord. The perineuronal pattern of PrPSc is also seen in the ventral horn neurons of the spinal cord and the dorsal root ganglion cells.[51]

In natural H-type BSE, granular, intraneuronal, linear, intraglial, and punctate PrPSc deposits in the brainstem are the most characteristic types. While mainly detected at the level of the DMNV, NST, NSTV, and in the reticular formation, a certain variability in PrPSc distribution has been described (Corona C. personal communication[53]) (Fig. 7). In experimental H-type BSE, large amounts of PrPSc are diffusely deposited throughout the CNS, including the cerebral cortex, basal ganglia, thalamus, hypothalamus, brainstem, and spinal cord. Fine or coarse particulate-type deposits in the neuropil of the gray matter throughout the brain and spinal cord are the most conspicuous type of PrPSc deposition. However, linear, perineuronal, and intraneuronal types of PrPSc staining can be observed in the cerebral cortex, basal ganglia, thalamus, and brainstem. Glial-type PrPSc deposition is

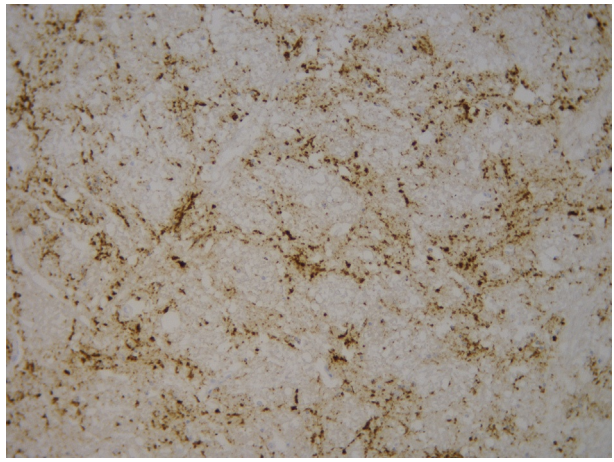

Fig. 7 IHC, pattern of PrPSc of H-type BSE, characterized by granular, intraglial, and small aggregates (20 ×).

predominantly identified in the cerebral cortex, basal ganglia, thalamus, hypothalamus, and hippocampus, and often in the cerebellar cortex, but it is not visible in the brainstem and spinal cord. Intraglial-type PrPSc deposition is highly consistent throughout the white matter of the CNS and spinal cord. Some animals show the presence of PrPSc-positive plaques scattered throughout the cerebral white matter.[50]

8. WESTERN BLOTTING

Western blotting is a widely used immunobiochemical technique for the diagnosis of prion diseases. Various different WB methods have been developed since the active surveillance system was instituted in Europe: some are used as screening tests and others to confirm the suspect cases identified by active and passive surveillance. The techniques are based on the immunodetection of the PrPSc at the level of the medulla oblongata. WB methods are very versatile since they can be applied on fresh, frozen, and autolytic tissues.[54] Comparable in diagnostic sensitivity to IHC techniques, WB remains the method of choice, along with IHC, for the confirmation of suspect BSE cases. A highly sensitive method, WB uses a large mass (2–4 g) of CNS material and several steps to concentrate PrPSc. Alternative less time-consuming and less costly methods are now employed by TSE Reference Laboratories in Europe to confirm BSE cases. Different from published methods, in-house tests for confirmatory testing must be validated; their analytical sensitivity, together with commercial tests, is continuously monitored through annual ring trials conducted by the TSE European Union Reference Laboratory (APHA, UK).

SAF-immunoblotting entails the preparation of homogenates from brainstem and digestion of the samples with proteinase K. After ultracentrifugation, the pellet is dissolved in Laemmli buffer, and an equivalent of 10 mg of wet tissue is loaded onto SDS-polyacrylamide gels. Following separation, the proteins are transferred onto a polyvinylidene difluoride (PVDF) membrane. PrPSc is detected by utilizing monoclonal antibody anti-PrP, and the presence of immunosignals is revealed by phosphatase-conjugated anti-mouse IgG developed using a chemiluminescence system and visualized on Hyperfilm ECL sheets. In positive test cases, confirmatory WB shows the presence of PrPSc characterized by an electrophoretic pattern consisting of three bands that correspond to the di-, mono-, and non-glycosylated forms, migrating at approximately 30, 25, and 19 kDa, respectively. No PrP signals are present in the bovine cases confirmed as negative

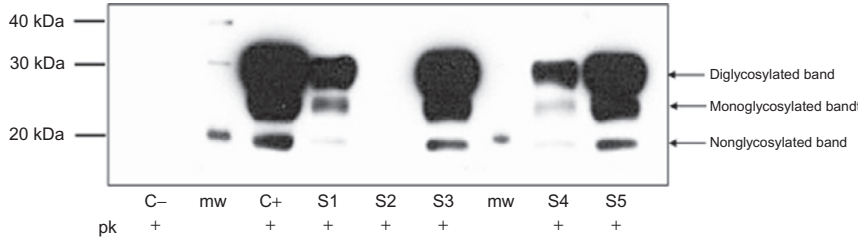

Fig. 8 Western Blotting analysis of positive and negative BSE cases. C-, negative BSE control; C+, positive BSE control; Lanes S1, S3, S4, and S5, positive BSE samples; lane S2, negative BSE sample; Mw, molecular markers. Immunodetection was performed by monoclonal antibody 6H4.

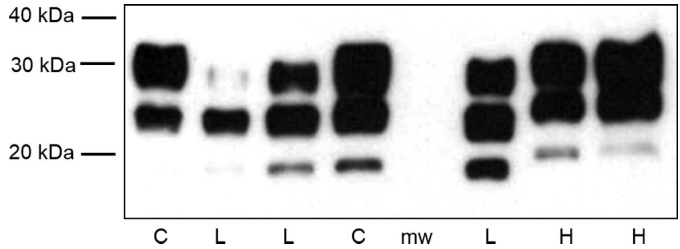

Fig. 9 Western Blotting analysis of PrPSc from classical and atypical BSE cases. C, classical BSE; L, low-type BSE; H, high-type BSE; Mw, molecular markers. Immunodetection was performed by monoclonal antibody 6H4.

since the PrPC is completely digested by proteinase K (Fig. 8). The application of immunoblotting methods is essential for evaluating the molecular features of PrPSc and so to discriminate between classical and atypical BSE isolates. The H-type is characterized by a significantly higher molecular size of the nonglycosylated PrPSc form and a conventional glycopattern, while the L-type or BASE has only a slightly lower molecular size of the nonglycosylated PrPSc form and a predominance of the monoglycosylated moiety (Fig. 9).

9. SCRAPIE-ASSOCIATED FIBRILS

Electron microscopy highlights BSE-associated fibrils, the bovine equivalent of SAF. The fibrils are composed of PrPSc and can be extracted from fresh, frozen or formalin-fixed nervous tissue by homogenization, centrifugation, and digestion with proteinase K, followed by coloring with phosphotungstic acid. Observation by electron microscopy reveals fibrils with a simple or double helix structure 100–500 nm in length.

10. IN VITRO AMPLIFICATION TECHNIQUES

A key challenge to coping with TSE diseases in humans, livestock, and wildlife is to develop sensitive and rapid high-throughput assays for the routine detection of prions and the early diagnosis of TSEs. Owing to the peculiar characteristics of prion diseases, such as the absence of a classical immune response and the absence of nucleic acids in the prion agent, many conventional methods for detecting infections by common pathogens cannot be applied. In fact, current biochemical or immunocytochemical assays have low sensitivity and may yield inconclusive results, rendering them unreliable in a clinical or preclinical setting of prion-infected hosts. In contrast, in vitro amplification techniques can be used to determine whether a tissue contains any prion seeding activity. Research in this direction has led to the development of reliable assays for the early diagnosis of prion disease in easily accessible tissues or body fluids. Both protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) rely on the detection of PrP structural conversion and polymerization upon the addition of PrPSc seeds contained in the infected samples. These methods are usually more sensitive than bioassays by two to three orders of magnitude, and endpoint titration can be performed in a format similar to the bioassay.[52]

10.1 Protein Misfolding Cyclic Amplification

In 2001, Soto and colleagues described a new type of in vitro prion conversion reaction called PMCA that greatly improved efficiency and sensitivity over the initial conversion reactions of the prion protein in cell-free environments (cell-free conversion assay).[55] PMCA combines cycles of sonication that result in hydrodynamic sharing of PrPSc aggregates into smaller nuclei. During incubation, the PrPSc molecules present in the nuclei imprint their abnormal conformation onto PrPC, which is subsequently incorporated into growing PrPSc aggregates. The sonication–incubation cycles are repeated to achieve exponential amplification of minute quantities of PrPSc present in a sample. In a typical PMCA reaction, brain extracts are used as a source of PrPC. The cyclic nature of the system and the possibility to refresh the substrate at each round enable the performance of as many cycles as needed to reach the amplification state needed for the detection of PrPSc in a given sample. In these conditions, the PrPSc molecules can be amplified to detectable levels by immunoblotting. PMCA is a promising

platform for prion diagnosis in biological tissues or fluid samples, including blood, urine, feces, or cerebrospinal fluid, where the level of PrPSc is estimated to be in the range of picograms per mL.[56] PMCA has been successfully employed to study prion behavior, the molecular mechanism of prion replication, the cellular factors involved in prion propagation, and the still unknown aspects of prion strains and their trans'species conversion characteristics upon passage. Thus, PMCA holds promise not only as a prion detection assay but also as a tool to investigate the mechanism of prion-induced PrP conversion. Despite the advances achieved with PMCA in prion research, the technique has several pitfalls: amplification of PrPSc in apparently normal individuals, spontaneous generation of misfolded PrPSc, and possible cross-contamination. Other limitations are the time necessary to achieve optimal sensitivity and the use of brain-derived PrPSc as the amplification substrate. Clearly, there was a need to develop an accurate, high throughput diagnostic that is automated and can be easily used in a routine diagnostic laboratory.

10.2 Real-Time Quaking-Induced Conversion

To overcome the technical complexities of PMCA reactions, a new practical prion assay, quaking-induced conversion (QuIC), developed by Atarashi and colleagues[57] substitutes sonication with automated tube shaking for the conversion of recombinant PrP (rPrP) substrates. QuIC-based methods use the rPrPSc produced in bacteria as a substrate for seeded polymerization and tube shaking to break the generated polymers and provide new seeds for conversion in amplification rounds. The rPrP-QuIC method also solves the problem of using brain-derived PrPSc as the amplification substrate. Moreover, the fact that rPrPSc can be easily mutated allows investigation into the role of specific sequences or amino acids in the conversion reaction and accelerates studies on the detection of prions. One of the major refinements of the technique was the substitution of WB with real-time fluorescent color reaction based on a fluorescent amyloid-sensitive thioflavin T dye (ThT). The formation of these prion-seeded amyloid fibers is detected in real time by reading ThT fluorescence over time. In its real-time and multiwell plate format, RT-QuIC has the potential for use in high-throughput screening of samples. The method provides for a test as quantitative and sensitive as in vivo testing[52] and it has been adapted to different types of TSE. The RT-QuIC assay allows rapid and highly sensitive discrimination between prion-infected and uninfected brain tissues. Furthermore, it has been proven sensitive for detecting prions in infected tissues and fluids, including

cerebrospinal fluid, saliva, nasal fluids, and blood.[57,58] The method does not detect prion infectivity in a given tissue, but rather allows for the detection of a seeding activity potentially associated with prion replication. Indeed, based upon the quantitative correlation between prion seed concentration and the lag time to the start of the conversion reaction, qRT-QuIC allows for the quantification of prion infectivity in tissues, body fluids, and excreta.[59] For quantification, the amplified PrPSc signal can be compared with that seen in endpoint titrated material run under the same conditions (such as brain homogenate from animals at the terminal stage of disease) or to PrP calibration curves. Like animal bioassays, the RT-QuIC assay can titrate the seeding activity in endpoint diluted samples.[52,57] Serial dilutions of a given sample are used as seeds, and the seeding dose (SD) giving 50% ThT-positive replicate reactions (SD50), i.e., the 50% endpoint dilution, is estimated. The SD50 is analogous to the 50% lethal dose (LD50) determined in an endpoint dilution animal bioassay. Moreover, RT-QuIC has several major advantages over animal bioassays, including practicality, high-throughput potential, rapidity, and lower cost. The quantitative aspect of qRT-QuIC suggests that it can provide reliable assessment of anti-prion therapy in vivo in order to follow the effects of therapy on the progression of prion diseases. Furthermore, since qRT-QuIC provides an ultrasensitive method for quantifying pathological amyloid aggregate seeds, it may also be applicable to other disease-associated proteins rich in β-pleated structures that bind ThT and show seeded aggregation. Some prion strain types are known to be fairly resistant to amplification by either PMCA or RT-QuIC. However, recent studies have adapted RT-QuIC assays for the sensitive detection and discrimination of C-BSE, L-BSE, and H-BSE (Fig. 10).[60,61] Testing with the RT-QuIC assay on brain tissue from cattle affected by BSE strains showed that all three forms can be detected and distinguished with the use of particular rPrPSc substrates. RT-QuIC tests have been adapted to detect many types of prion seeding activity; however, numerous critical issues remain: the evaluation of outcomes for the antemortem diagnosis of TSE in animals of farm interest from which the least invasive and most economical biological peripheral matrix can be identified for prion testing in living subjects; the recognition of classical and atypical forms of PrPSc by a unique protocol in order to obtain a single diagnostic assay for TSE diseases; the detection of PrPSc from biological fluids by removing soluble components that could inhibit the assay; the collection of preclinical and clinical data from subjects testing PrPSc-positive in peripheral matrices to better define the distribution of peripheral TSE infectivity.

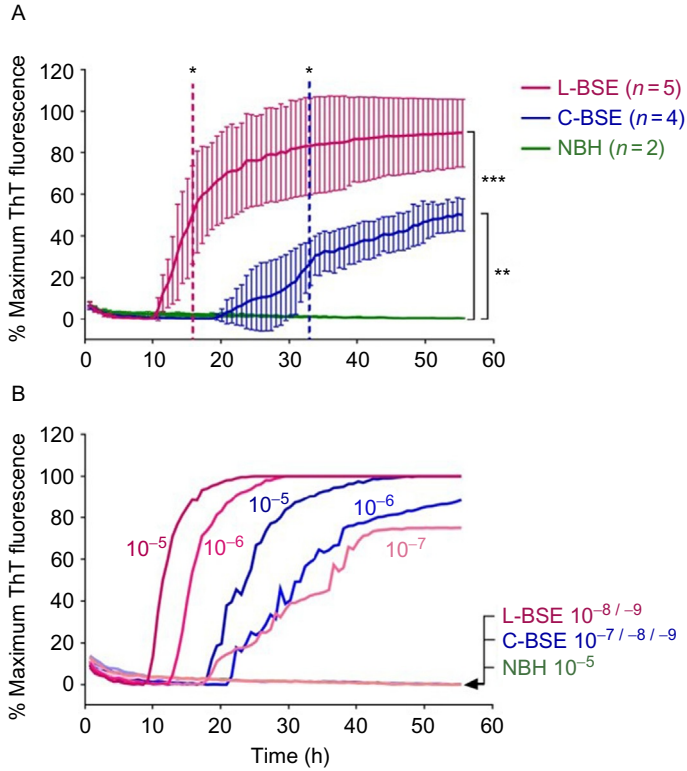

Fig. 10 *RT-QuIC sensitivity for C-BSE and L-BSE detection*. (A) L-BSE-infected (*magenta*), C-BSE-infected (*blue*), or normal negative control (NBH, *green*) 10^{-5} brain tissue dilutions were used to seed quadruplicate RT-QuIC reactions using the Ha-S rPrPSen substrate. (B) Serial dilutions (10^{-5} to 10^{-9}) of C-BSE-infected or L-BSE-infected brain tissue or a 10^{-5} dilution of uninfected brain tissue were used to seed quadruplicate RT-QuIC reactions with Ha-S rPrPSen as the substrate. The data show the average ThT fluorescence of four replicate wells. Each ThT reading is indicated as the percentage of the maximum value achievable by the plate readers as a function of reaction time. Square brackets signify the difference between the text groups and the control at the 55-h endpoint. $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.

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