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Chapter 1

Classical and Atypical Bovine Spongiform Encephalopathy: Epidemiology, Pathogenesis and Diagnosis

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Additional information is available at the end of the chapter

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Abstract

Classical bovine spongiform encephalopathy (C-BSE) is a fatal neurodegenerative disease of cattle, detected in the United Kingdom and many other countries since the 1980s. The origin of C-BSE is uncertain, but epidemiological studies suggest that the source of this disease was cattle feed prepared from prion-infected animal tissues. To date, cattle populations have been monitored through passive and active surveillance programs. From 2004, two different forms of BSE termed as L-BSE, also known as bovine amyloidotic spongiform encephalopathy (BASE), and H-BSE have been discovered in Italy and France. All these atypical cases have been detected in animals over 8 years of age. To date, there is no comprehensive information about the origin of the atypical BSEs (sporadic vs. acquired). Moreover, there are only very limited data available, concerning the pathogenesis of both atypical forms, as compared to C-BSE. This chapter provides 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 the routine diagnosis based on rapid tests, histological, immunohistochemical, and Western blot examinations.

Keywords: brainstem, C-BSE, atypical BSE, neurodegenerative diseases, surveillance, rapid tests, confirmatory tests

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1. Introduction

Classical bovine spongiform encephalopathy (C-BSE) is a fatal neurodegenerative disorder of cattle that belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs). C-BSE is characterized by the accumulation of a disease-associated abnormal form of prion protein (PrPSc) in the central nervous system (CNS). PrPSc is commonly accepted as the pathological agent of TSEs and may be a post-translationally modified form of a normal cellular prion protein (PrPC). C-BSE has a long incubation period, about 2.5–8 years, with clinical disease usually affecting adult cattle at a peak age onset of 4–5 years, and with all breeds being equally susceptible. C-BSE is characterized by altered behavior and uncoordinated movement; histopathologic features include neuronal and neuropil vacuolization, glial reaction, and the complete absence of inflammatory lesions. BSE was first described in the United Kingdom (UK) in 1986 and is now classified as a potentially lethal zoonotic disease acquired via contaminated food [1–3], although the definite origin of BSE is still unknown. In 1996, the evidence of the pathogenetic relationship between BSE and a fatal neurodegenerative disorder in human (now known as the variant Creutzfeldt-Jakob disease) emerged and attracted the concern of the public.

From the UK, C-BSE spread 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 United Kingdom, 5,500 in Europe, and 60 cases in the rest of the world (Brazil, Canada, Israel, and Japan). The World Organisation 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.

Two different atypical BSE strains in cattle were discovered in 2004 in Italy and in France, respectively [4, 5]. However, these strains have been also identified in others European countries, Japan, and the Americas. They were designated L-type and H-type due to the molecular weight of PrPSc after protease degradation and Western blot (WB) analysis. The L-type is also known as bovine amyloidotic spongiform encephalopathy (BASE) because of the presence of PrP-positive amyloid plaques in the brain.

The origins of atypical BSEs remain obscure, and it has, therefore, been postulated that they represent a spontaneous TSE in cattle, comparable to most of sporadic CJDs cases in man. They are mainly detected in cattle that are 8 years of age or older. Most cases were identified in fallen stock and none were reported as clinical suspect, which suggested that the clinical presentation is unlike C-BSE.

Data on atypical BSE cases reported in the EU BSE databases since 2001 show that a total of 44 cases of L-type and 60 of H-type BSE have been identified in Europe. The prevalence of atypical BSE cases in the rest of the world is unknown because there are no official surveillance requirements or systematic reports from different countries.

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2. Epidemiology

Epidemiology played a key role in deepening the knowledge of the dynamics and features of the disease and represented the foundations on which the initial control measures were built, in the early stages of the BSE epidemic. Epidemiological data established the following: dairy cows had a higher risk of BSE compared to beef cows, as demonstrated later [6–9]; BSE has an aetiological similarity with scrapie; the cases were geographically scattered in the UK, except for Scotland (it was later found that the rendering system procedures remained unchanged) where no cases were reported in the first period of the epidemic; all sick animals were index cases—that is there was usually one case per herd; a shape typical of an extended common source [10].

In the 1986–1996 decade, the British trade in livestock and animal by-products dropped sharply and the question as to whether BSE could be present in other countries arose. Shortly after reports of BSE cases appeared throughout Europe.

Following the BSE crisis, the European Parliament requested the European Commission (EC) to review its advisory system for public and animal health issues, especially those related to agricultural production and food. The EU Scientific Steering Committee (SSC) was appointed to supervise eight other specialized scientific committees dealing with food safety and public and animal health [11]. The SSC appointed a working group on TSEs to assess the risk that a country could have undetected BSE cases within its own bovine population. The outcome was the Geographical BSE-risk assessment (GBR).

2.1. Geographical risk assessment

The Geographical BSE-risk assessment (GBR) is an indicator of the likelihood in the presence of one or more bovines being infected with BSE, preclinically as well as clinically, at a given point in time, in a country/region. It is based on a semiquantitative analysis of the likelihood that the BSE agent was introduced into a country/region and if so when and to what extent and the potential of it being recycled and potentially amplified or eliminated [12].

The BSE/cattle system model is influenced by the external challenge by import of BSE contaminated meat and bone meal (MBM) and/or BSE incubating cattle. If this cattle system is unstable, allowing recycling and amplification of the infectious agent, the epidemic will grow. The system is characterized by its capability to prevent an external threat (challenge) and its ability to remove BSE infected cattle and/or MBM before processing (stability).

2.2. Risk factors

The assessment of the risk factors was biased in the early stages of the epidemics, due to the lack of an accurate surveillance. Subsequently, an enforced active surveillance system was put in place in 2001 in the majority of the European countries in order to carry out a more reliable analysis.

The main risks factors can be summarized as follows:

Production type and herd size play an important role in the spreading of the infection, albeit inconsistently across countries [8, 13–15].

A higher amount of compound feed leads to a higher risk of infection; the risk is higher in very large dairy farms because of the greater use of such feed.

Some studies demonstrated a higher incidence of the disease in autumn-born cattle compared to spring-born ones [16–18].

The relationship between milk yield and the risk of BSE was studied [7]; however, milk yield is only an approximation of the amount of concentrates given to cattle.

Dairy cattle have a higher risk than beef suckler herds [8, 18].

In July 1988, the ban on feeding ruminant-derived MBM to ruminants was introduced; a study on a cohort of cattle born after the ban, the area-level BSE risk was additionally associated with greater numbers of pigs per area relative to cattle [19]. These findings supported the influential role of low-level cross-contamination of cattle feed by pig feed in BSE incidence as the epidemic evolved.

2.3. Epidemiologic surveillance

The OIE established the criteria by which the BSE status of a country should be determined. These criteria are: the result of risk assessment, the implemented measures to manage the BSE risk, and the reported incidence rates (the OIE recommended an intensive passive surveillance approach).

An updated version of the BSE chapter was approved by the 68th OIE General Session in May 2000; this states that the presence of the disease can only be determined on the basis of the following criteria:

- Risk assessment that includes MBM consumption, importation of MBM, importation of potentially infected animals or ova/embryos, epidemiological situation of TSEs in a country, level of knowledge of the livestock structure, and source of animal waste, parameters of treatment and methods of production;
- Continuous awareness programs for veterinarians, farmers, and any other involved professional;
- Mandatory notification and diagnostic testing of all cattle showing clinical signs consistent with BSE;
- Continuous monitoring systems that take into account the risk factors listed and meet the criteria defined in a special appendix to the text;
- Diagnostic examination of the sample in an approved laboratory.

In January 1999, Switzerland enforced a targeted active surveillance program for BSE, performing a specific Western blot test [20] on all emergency slaughtered adult cows and on all fallen stocks. The newly enforced plan allowed us to identify a higher number of cases,

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50% of which would have been missed with passive surveillance alone. This finding led the European Commission (EC) to quickly approve the use of various rapid tests to detect the PrPSc protein in CNS tissue samples.

Over the past decade, European legislation setting the minimum age of cattle at which rapid testing is to be performed reflects, in part, the dynamics of the BSE epidemic, with a progressive extension of the cutoff age.

Regulation (EC) 999/2001 [21] is the legislative response to the BSE crisis to prevent, control, and eradicate the disease and became a legal instrument in 2001. It provides a harmonized set of rules to be implemented in all member states. The rules are very strict because that what was necessary to get on top of the disease. The regulation is built on two main pillars, (i) a total feed ban which outlawed the feeding of animal proteins to all farmed animals and (ii) removal of specified risk material (SRM) from all slaughtered animals (bovines and small ruminants). Other prevention and control measures contained in the regulation are the surveillance measures:

- Before 2001: only passive surveillance (notification of cattle showing clinical symptoms consistent with BSE).
- From 1 January, 2001 [21] mandatory testing in healthy slaughtered animals aged above 30 months and in animals at risk above 24 months of age (fallen stock, emergency slaughter and animals with clinical signs at the ante mortem).
- From 1 January, 2009 [21]: all animals above 48 months (for 17 member states).
- From 1 July, 2011 [21]: healthy slaughtered animals above 72 months; animals at risk above 48 months (for 25 member states).

As of July 2013, the rapid tests for the detection of BSE cases have been suspended for the animals regularly slaughtered, and maintained for the animals above 48 months of age in at risk streams (fallen stock, emergency slaughtered and with clinical sign at *ante mortem*).

The introduction of the comprehensive active surveillance plan in 2001 marked a turning point in the capability to accurately describe the geographical distribution of BSE and the trend of the epidemic across Europe. Within a few months, the epidemiological BSE pattern (previously based on data only from passive surveillance) radically changed shape when reports of the disease unexpectedly arrived from countries other than the UK. Contrary to what was happening in Britain, where the number of BSE cases decreased by one-third (from 2301 to 1443) between 2000 and 2001, the total number of cases doubled (from 515 to 1012) in the rest of Europe and Japan also reported its first three cases. However, at least in Western Europe, 2001 also marked the beginning of the slow decline of BSE, demonstrating the efficacy of the control measures put in place since the mid-1990s: the MBM ban and the exclusion of specified risk material (SRM) from food and feed chains to mitigate the risk of exposure to infection.

Active surveillance subsequently allowed the identification of atypical forms which are probably very rare, different diseases, with epidemiological features different from those of classical BSE.

In the years to follow, the trend of C-BSE and, thus, the effectiveness of enforcement, would be monitored through the testing of huge numbers of cattle (10 million cows per year on average in the European Union). Now, some 15 years later, the problem seems to be solved, and since 2005 the European Union has been setting up an exit strategy from the crisis [22], providing for the gradual easing of the measures.

On the basis of the data collected from the information systems in place, and available from the annual reports the European Commission published between 2002 and 2014, detailed information can be gleaned about the prevalence and incidence of BSE in Europe and the temporal and geographical distribution of the disease.

3. Pathogenesis of classical and atypical BSE

The pathogenesis of C-BSE in cattle has been extensively studied, although there are still a number of knowledge gaps. After oral exposure to infective material, how prion agent crosses the epithelium is not exactly defined, but the most likely mechanism is via M-cells, a cell type present in the follicle-associated epithelium of the gut and tonsil which specializes in the transport of macromolecules across the epithelium [23]. These cells are capable of transcytosing 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) in gut-associated lymphoid tissue (GALT) of the ileocaecal junction and the jejunum and in Peyer's patches of the ileum [24].

Moreover, at 6–10 mpi the infectivity is also located in palatine tonsils [25]. At 12 mpi, a peak of infectivity in the distal ileum is related to the number of follicles involved and the amount of PrPSc detectable in the follicular dendritic cells (FDC) and TBM, indicating an increased clearance activity of these cells. There is a second peak of infectivity at 24 mpi, where PrPSc is mainly located in TBM and FDC of jejunum and ileum and, later, a third peak of PrPSc accumulation between 32 and 40 mpi [26].

During the infection of the gut, the TSE agent can come into contact with the fine nerve fibers of the mucosal plexus of the enteric nervous system [27]. Then, through mesenteric nerves, prion proteins accumulate in the cranial coeliaco mesenteric 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 equine [28]. From the spinal cord, PrPSc then accumulates in the dorsal root ganglia, trigeminal, and cervical ganglia [29] and the adrenal glands and sciatic nerve have also been described as positive tissues with demonstrable prion protein accumulation [30]. Between 42 and 84 mpi PrPSc spreads to the spindles of various muscles such as the masseter, the triceps brachii, intercostal muscles, and the semitendinosus [31].

Currently, it is very difficult to hypothesize about the pathogenesis of atypical BSE, because information on the tissue distribution of PrPSc in cattle affected by atypical BSE is limited, and largely confined to experimental animals at clinical endpoint. According to experimental transmission studies, PrPSc has been reported in CNS tissues, peripheral ganglia and nerves, muscles (muscle spindles), adrenal glands, and retina for both H-BSE and L-BSE [32]. 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 the possibility that prions propagated in the CNS and were spread centrifugally by nerve pathways [33].

4. Diagnosis

Historically, BSE diagnosis has always been made by the detection of characteristic vacuolation in certain anatomical regions of the formalin-fixed brain [34] by histopathological examination. In the early 1980, the discovery of scrapie-associated fibrils (SAF) and the production of antibody against SAFs were the first steps of the revolution in TSE investigations [35, 36]. At the moment, cattle populations are monitored through passive and active surveillance programs. Under passive surveillance, cattle are tested for the disease with confirmatory tests: histopathology (H&E), immunohistochemistry (IHC), Western blot (WB), or demonstration of characteristic fibrils (SAF) by electron microscopy. If a brainstem sample tests negative, the OIE manual requires that the entire brain of the animal be tested to establish differential diagnosis. Samples collected in active BSE surveillance are screened with approved rapid tests, in accordance with Regulation (EC) No. 999/2001 (European Commission, 2001) on the prevention, control, and eradication of certain TSEs. In inconclusive or positive cases, the sample is submitted to confirmatory tests.

5. Sampling

The first stage of all the current TSE diagnostic or screening tests involves the sampling of the CNS and the subsequent examination of the sampled tissue for the presence of PrPSc. In particular, the minimum sampling requirement is the brainstem, at the level of the obex [37]. This area can be accessed through the foramen magnum using a proprietary sampling spoon (**Figure 1**).

The quantity of tissue taken for testing (**Figure 2**) should be sufficient to provide the following:

A hemisection of fresh obex, for the initial rapid test.

A fixed cross-section, or hemi-section of obex for confirmatory IHC and H&E.

Sufficient remaining fresh-frozen medullary tissue (adjacent to the obex) for primary molecular testing (discriminatory WB) and possibly a range of secondary and tertiary testing (5–10 g whenever possible).

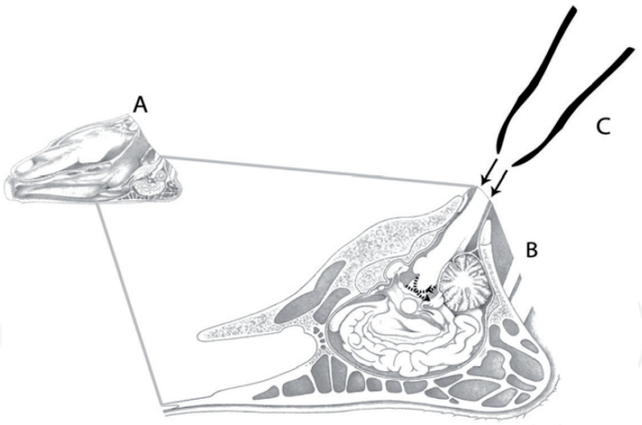


Figure 1. A) Place the disarticulated head upside down and C) using the forceps, B) insert it to the level of the foramen magnum.(http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/2.04.06_BSE.pdf)



Figure 2. Fresh brainstem, at the level of the obex.

6. Rapid tests

Rapid molecular diagnostic assays became officially available in the late 1990s. With the enforcement of Regulation (EC) No. 999/2001 [21] the use of rapid tests became mandatory: many countries subsequently detected the first BSE cases. To provide dependable tools for

an active surveillance system, in 1999 the EC carried out the first scientific evaluation of four new rapid *post mortem* BSE tests to assess their diagnostic accuracy and analytical sensitivity on brain tissue from clinically affected bovines [38]. Subsequent EU validation exercises enhanced the estimating parameters, including test robustness on autolyzed samples and testing of negative field samples to address the test specificity and to simulate routine activity [39–41]. To date, the EC has assessed 19 rapid tests in the frame of 3 "successive" evaluations and approved 9 for survey purposes [42]. 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 [43]. The analytical sensitivity study was then evaluated by the European Food Safety Authority (EFSA) [44, 45] on the basis of current EFSA requirements for the evaluation of TSE rapid *post mortem* tests [46]. In that context, the lowest limit of detection (LOD) of rapid tests approved for the diagnosis of classical BSE in bovines was assessed. The rapid tests with an LOD poorer than $2\log_{10}$ as compared to the best-performing assay could not be recommended for use in the frame of BSE monitoring in cattle within the EU. Recent studies [47] demonstrate the suitability of BSE EU-approved rapid tests also for the detection of both L- and H-type BSE. Despite the evidence of clear differences in relative analytical sensitivity, the LOD of rapid tests applied on BSE atypical nervous tissues meets EFSA criteria for BSE monitoring purposes.

According to EU Regulation 999/2001, for the purposes of carrying out the active only the following methods shall be used as rapid tests for the monitoring of BSE in bovine animals:

The immuno-blotting test based on a Western blotting procedure for the detection of the Proteinase K-resistant fragment PrPSc (Prionics®—Check WESTERN Prionics AG, Schlieren-Zurich, Switzerland).

The microplate-based immunoassay for the detection of PrPSc (TSE Version 3, Enfer Scientific®, Newhall, Naas, County Kildare, Ireland).

The sandwich immunoassay for PrPSc detection (short assay protocol) carried out following denaturation and concentration steps (TeSeE™ Purification-Detection SAP Test Kit, Bio-Rad Laboratories, Marnes-La-Coquette, France).

The microplate-based immunoassay (ELISA) which detects Proteinase K-resistant PrPSc with monoclonal antibodies (Prionics®—Check LIA BSE Antigen Test Kit, Prionics AG, Schlieren-Zurich, Switzerland).

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 & IDEXX® HerdChek BSE-Scrapie Antigen Test Kit, EIA, Westbrook, ME, USA).

The lateral-flow immunoassay using two different monoclonal antibodies to detect proteinase K-resistant PrP fractions (Prionics®—Check PrioSTRIP, Prionics AG, Schlieren-Zurich, Switzerland).

The two-sided immunoassay using two different monoclonal antibodies directed against two epitopes presented in a highly unfolded state of bovine PrPSc (BetaPrion® BSE EIA Test Kit, AJ Roboscreen, Leipzig, Germany).

A part from the *Prionics®* Western blotting and lateral-flow immunoassay, the remaining approved tests are based on semi-quantitative ELISA methods that produce a qualitative result relative to a cutoff value. They include a PK digestion step to unmask cryptic epitopes, except for the *IDEXX HerdChek® BSE-scrapie EIA*, which relies on conformational detection technology using a specific aggregate specific capture ligand on a dextran polymer (Seprion ligand technology, Microsens Biotechnologies, London, UK) [48]. The lateral-flow immunoassay *Prionics®—Check PrioSTRIP* produces results that can be interpreted by a computerized *PrioSCAN®* software to minimize subjectivity, although a visual interpretation by two independent readers is also validated. The *Prionics®—Check Western* is based on a Western blotting procedure. It is both a qualitative and quantitative test, as it distinguishes PrPSc in non-, mono-, and diglycoforms while expressing their respective quantitative ratio and migration positions. The diagnostic criteria for positive results are based on the exhibition of a three-band signal, the top one corresponding to a protein with an approximate molecular weight of 30 kD. Signal intensity decreases from top to bottom, but the higher band should be clearly visible immediately under the PK band.

7. Histopathological examination

The histological examination is performed on formalin-fixed, paraffin-embedded brain sections that are stained with hematoxylin and eosin (H&E). The histological C-BSE changes in the CNS, that are visible using an optical microscopy, are vacuolation of gray matter neuropil (spongiform change) and/or vacuolation of neurons, with a predilection for certain neuroanatomic locations [34, 49]; astrocytosis and neuronal degeneration may also be present [50]. The target areas at the level of the obex for the diagnosis of BSE are the solitary tract nucleus (NST) and the spinal tract nucleus of the trigeminal nerve (NSTV) (**Figures 3** and **4**). Moreover, vacuolation could be also present in central gray matter of the midbrain and mild spongiform changes of the neuropil could be observed in some cattle 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 [4]. In experimental cases, a more severe involvement of central gray matter (periaqueductal gray) and rostral colliculus but not the vestibular nuclear complex is observed. Additional brain areas, including the olfactory areas, amygdalae, hippocampi, and dorsal horns of spinal cords, are severely involved. Ventral and dorsal roots do not show major pathological changes [51]. In experimental H-type BSE, vacuolar changes are generally observed in all the brain areas. The major vacuolation appears in the thalamic nuclei and neuropil of the central gray matter of the

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midbrain, and mild vacuolation is found in the caudal cerebral and cerebellar cortices. In the vestibular and pontine nuclei, spongy changes are not as prominent as in the other brainstem nuclei [52].

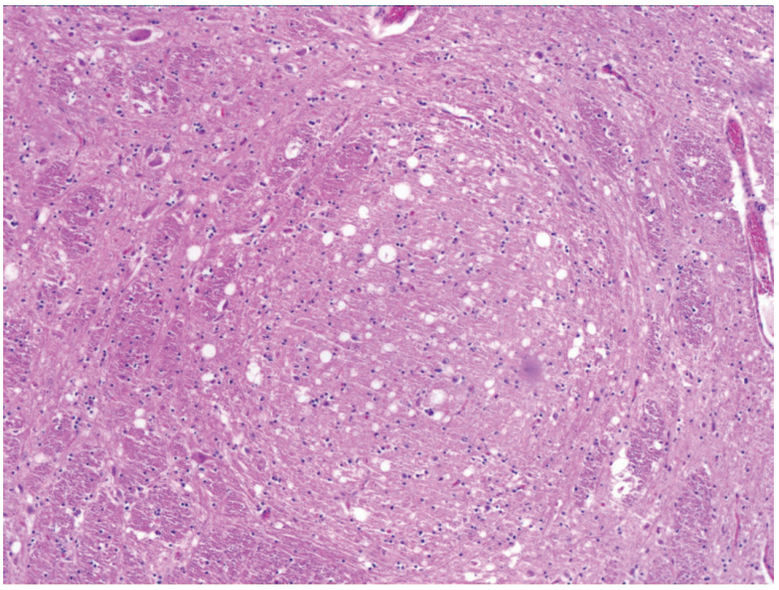


Figure 3. H&E, nucleus of the solitary tract of C-BSE: presence of spongiosis in the neuropil (20X).

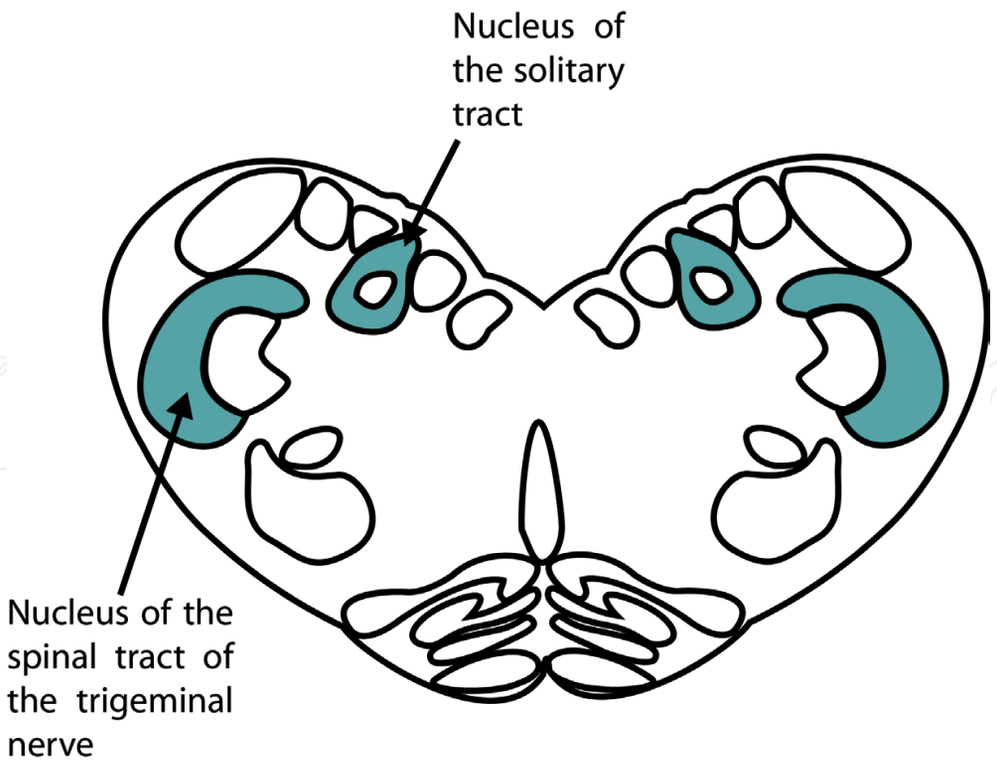


Figure 4. Section of the obex showing the target nuclei for BSE diagnosis.

8. Immunohistochemistry

Immunohistochemical analysis is performed on paraffin-embedded brain tissues in order to highlight the presence of PrPSc accumulation. The samples are deparaffinated, rehydrated, pretreated with 98% formic acid, and autoclaved at 121°C, then incubated at 4°C with monoclonal primary antibody, incubated with avidin-biotin-peroxydase, reacted with chromogen 3-3′ diaminobenzidine (DAB), and counterstained with Mayer's hemalum. Different immunohistochemical types of PrPSc deposition can be observed in the brain of C-BSE-affected cattle [53]: glial type labeling with PrPSc deposits branching out from the nucleus of glial cells on their processes conferring them a stellate appearance is predominantly in central gray matter and cerebral lamina and also within medial pontine nuclei in cerebral cortex, thalamus, and obex; a granular type that is characterized by granular PrPSc accumulations in the neuropil is commonly found in the neuropil of gray matter nuclei such as dorsal motor nucleus of vagus nerve (DMVN), NST, and in thalamic nuclei; intraneuronal type with PrPSc immunoreactivity throughout the neuronal cytoplasm is often observed in DMVN, reticular formation, olivary nuclei, vestibular complex, pontine, and thalamic nuclei and hypothalamus; perineuronal type, consisting in PrPSc deposits around individual neuronal perikarya and neuritis in caudate and putamen nuclei of basal ganglia and in DMVN; linear tract characterized by PrPSc deposits along neuronal processes in particular at the level of reticular formation of the brainstem; coalescing type seemingly arising from the merging of granular PrPSc deposits to form amorphous or mesh-like masses and intraglial type with fine punctate PrPSc adiacent to glial nuclei (**Figure 5**).

In the matter of natural cases of BASE, a distinctive feature is the presence of PrPSc deposition prevalently in the more rostral portions of the brain rather than occurs in C-BSE. At the level of the brainstem, the prevalent PrPSc deposition patterns are the punctate and granular



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

types, which are mildly present in the hypoglossal and olivary nucleus and moderately present at the level of DMVN nucleus, NST, NSTV, and reticular formation [54]. Glial, intraneuronal, perineuronal, and linear tracts are also frequent in BASE cases in different brain areas. Another characteristic of BASE cases is the presence of PrP-positive amyloid plaques; 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. They are predominantly located in the thalamus, subcortical white matter, in deeper layers of cerebral cortexes and in the olfactory bulb (**Figure 6**) [4]. In experimental BASE cattle, abundant amyloid PrP-plaques are observed in subcortical white matter and in deep gray nuclei, as observed in natural BASE cases. No PrP-plaques are seen in the olfactory glomeruli, the cerebellum, or the spinal cord. Perineuronal pattern of PrPSc is also seen in ventral horn neurons of the spinal cord and in the dorsal root ganglion cells [51].

As regards natural H-type BSE, in the brainstem, granular, intraneuronal, linear, intraglial, and punctate PrPSc deposits are the most characteristic types, mainly detected at the level of the DMVN, NST, NSTV, and in the reticular formation; however, there is some variability in PrPSc distribution among different H-BSE cases [54] (**Figure 7**). Regarding experimental H-type BSE, large amounts of PrPSc are diffusely deposited in the cerebral cortex, basal ganglia, thalamus, hypothalamus, brainstem, and spinal cord. The most conspicuous type of PrPSc deposition is fine or coarse particulate-type deposition in the neuropil of the gray matter throughout the brain and spinal cord. Linear, perineuronal, and intraneuronal types of PrPSc staining are observed in the cerebral cortex, basal ganglia, thalamus, and brainstem. Glial-type PrPSc deposition is predominantly identified in the cerebral cortex, basal ganglia, thalamus, hypothalamus, and hippocampus and often in the cerebellar cortex, but is not

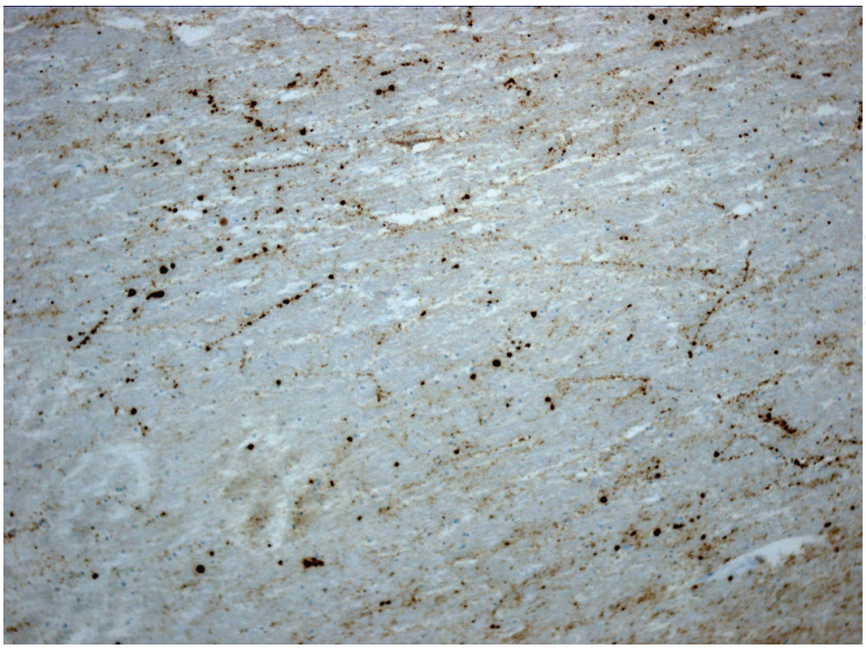


Figure 6. IHC, patterns of PrPSc of L-type BSE (BASE), characterized by amyloid plaques, granular deposits, and linear tracts (10X).

visible in the brainstem and spinal cord. Intraglial-type PrPSc deposition is very 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 [52].

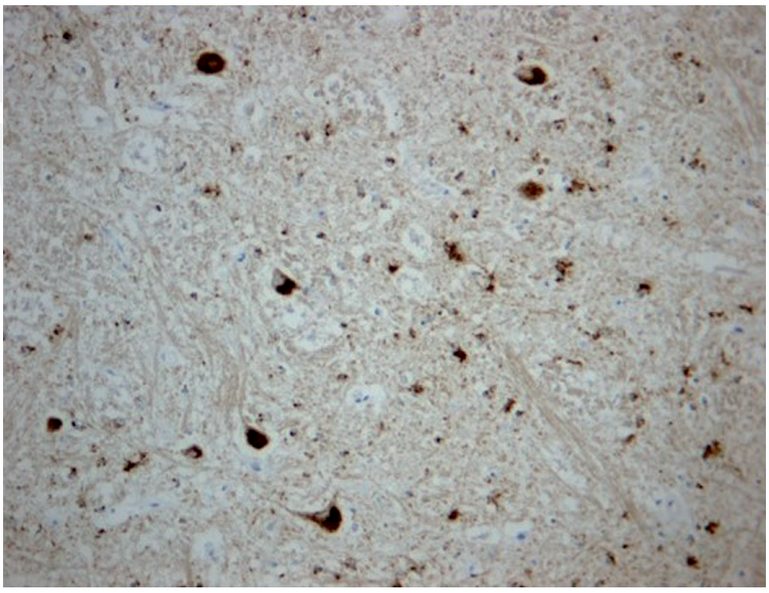


Figure 7. IHC, pattern of PrPSc of H-type BSE, characterized by intraneuronal deposits (10X).

9. Western blotting

The WB is an immunobiochemical technique widely used for the diagnosis of the prion diseases. Different WB methods have been developed since it was instituted active surveillance system in Europe, some used as screening tests and other to confirm the suspect cases identified by active but also through passive surveillance. These 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 tissue [55]. The SAF-immunoblot was the first such method for use in BSE diagnosis. It has similar diagnostic sensitivity to the IHC techniques, and remains the method of choice, along with IHC, for the confirmation of suspect BSE cases. It is a highly sensitive method using a large mass (2–4 g) of material and several steps to concentrate PrPSc. Alternative less time-consuming and less costly methods are now available in the different TSE Reference Laboratories in Europe to confirm the BSE cases. Unlike the published methods, in-house test applied for confirmatory purposes must be validated and their analytical sensitivity, together with the commercial tests, is continuously monitored by the TSE European Union Reference Laboratory (APHA, UK) through annual ring trial. The protocol of SAF-immunoblotting includes briefly the preparation of the homogenates from brainstem and the digestion of the samples with proteinase K. After ultracentrifugation step, the pellet is dissolved in Laemmli Buffer and an equivalent of 10 mg

of wet tissue is loaded on SDS-polyacrylamide gels and, after separation, proteins are transferred onto PVDF membrane. The detection of PrPSc is performed by monoclonal antibody anti-PrP and the presence of immunosignals is revealed by a phosphatase-conjugated anti-mouse IgG, developed using a chemiluminescence system and visualized on hyperfilm ECL. In the positive cases, the confirmatory WB shows the presence of PrPSc characterized by an electrophoretic pattern consists of three bands and corresponding to the di-, mono- and nonglycosylated forms, migrating at approximately 30, 25, and 19 kDa, respectively. No PrP signals are present in the bovine cases confirmed as negative since the PrPC is completely digested by proteinase K (**Figure 8**). The application of immunoblotting methods is very important to evaluate also 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 and a predominance of the monoglycosylated moiety (**Figure 9**).

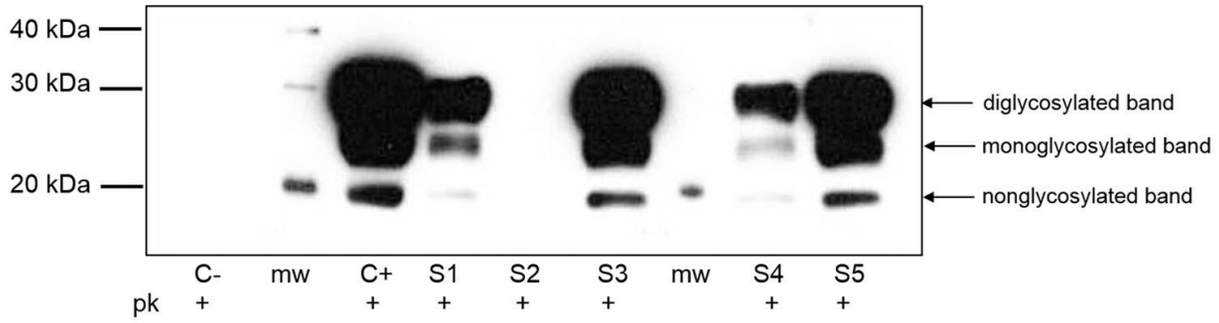


Figure 8. Western blotting analysis of positive and negative BSE cases. C–: negative BSE control; C+: positive BSE control; lanes S1, S3, S4 and S5: positive BSE samples; lane S2: negative BSE sample. Mw: molecular markers. Immunodetection was performed by monoclonal antibody 6H4.

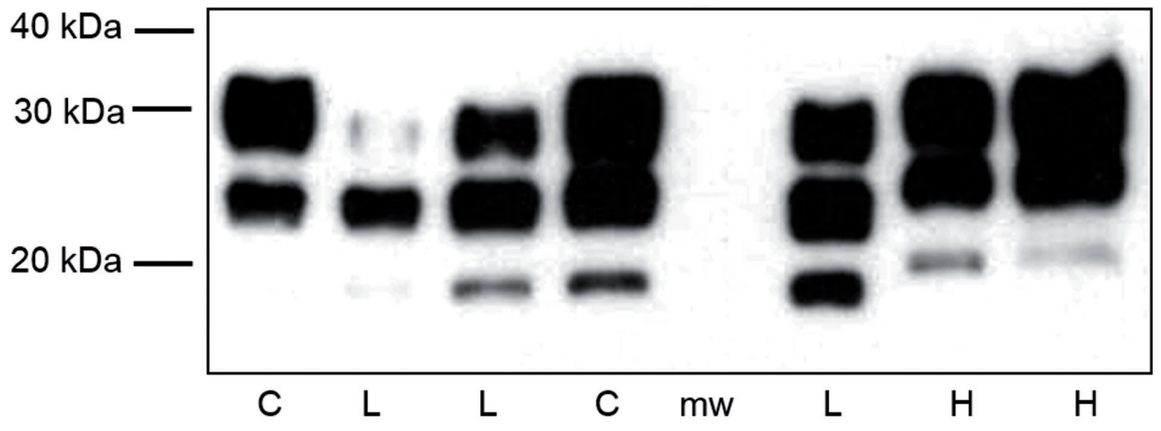


Figure 9. Western blotting analysis of PrPSc from classical and atypical BSE cases. C: classical BSE; L: low-type BSE; H: high-type BSE. Mw: molecular markers. Immunodetection was performed by monoclonal antibody 6H4.

10. Scrapie-associated fibrils

Electron microscopy highlights the BSE-associated fibrils, the bovine equivalent of SAF. The fibrils are composed of PrPSc and they are extracted from fresh, frozen, or formalin-fixed nervous tissue with the use of a homogenization treatment, differential centrifugations and digestion with proteinase K and colored with phosphotungstic acid. The observation to the electron microscopy allows us to highlight the fibrils with simple or double helix structure of 100–500 nm of length.

11. *In vitro* amplification techniques

A major problem for the effective management of animal prion diseases is the lack of rapid high-throughput assay to detect low levels of prions for the ante-mortem diagnosis of these diseases. In prion-affected animals, PrPSc is detected in a variety of peripheral tissues and body fluids, including blood, urine, saliva, cerebrospinal fluid (CSF), and nasal fluids; however, a validated diagnostic test is not available, yet. Current biochemical or immunocytochemical assays are roughly sensitive and might provide inconclusive results and consequently not reliable in a clinical or a preclinical setting of prion infected hosts in contrast to *in vitro* amplification techniques that can be used to determine whether a tissue contains any prion seeding activity. Two very efficient procedures to amplify prions in a test tube have emerged in the last decades, such as protein misfolding cyclic amplification (PMCA) or real-time quaking-induced conversion (RT-QuIC). Both depend on the detection of PrP structural conversion and polymerization upon addition of PrPSc "seeds" contained in the infected samples. These methods are usually more sensitive than the bioassay by two to three orders of magnitude, and endpoint titration can be performed in a format similar to the bioassay [56].

11.1. Protein misfolding cyclic amplification

In 2001, Soto and colleagues described a new type of *in vitro* prion conversion reaction called PMCA which greatly improved the efficiency and sensitivity compared to the initial conversion reactions of the prion protein in cell-free environment (cell-free conversion assay) [57]. The prion amplification by PMCA is based on repeated cycles of incubation and sonication during which increasing multimers of PrPSc are fragmented by sonication to induce formation and increase the effective concentration of PrPSc aggregates. In the typical reaction of PMCA, 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 enables the performance of as many cycles as required to reach the amplification state needed for the detection of PrPSc in a given sample. In these conditions, the PrPSc can be amplified to detectable levels by immunoblotting. PMCA allows the detection of minute amounts of PrPSc in biological tissues or fluid samples including blood, urine, feces, or cerebrospinal fluid from many prion-infected species. This method has sufficient sensitivity for PrPSc detection in blood in the asymptomatic stages of prion diseases [58]. In recent years, this method has become very useful to study different aspects of the prion protein such as to understand the molecular mechanism of prions' replication, the cellular factors involved in the propagation of the prions, and the still unknown aspects related to the prion strains and their trans-species conversion characteristics upon passage.

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Thus, PMCA has promise not only as a prion detection assay, but also as a tool to study the mechanism of prion-induced PrP conversion. Despite the progress that PMCA has facilitated in prion research, the fact that the amplification process to detect prions relies on sonication, makes it difficult to control. In addition, the limitations of PMCA include the time required to achieve optimal sensitivity and the requirement for brain-derived PrPSc as the amplification substrate. As a result, there was a need to develop an accurate, high throughput diagnostic that is automated and can be easily used in a routine diagnostic lab.

11.2. Real-time quaking-induced conversion

To avoid technical complexities associated with PMCA reactions, a new practical prion assay, quaking-induced conversion (QuIC), has been developed by Atarashi et al. [59]. The QuIC method uses recombinant prion protein (rPrPSc) produced in bacteria as a substrate for seeded polymerization and shaking instead of sonication is performed to break the generated polymers and provide new seeds for conversion in amplification rounds. As in the amyloid seeding assay, ASA, polymerization of rPrPSc into amyloid fibers can be detected by a fluorescence shift in the dye thioflavin T (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, the RT-QuIC has the potential to be used for the high throughput screening of samples. Since bacterially expressed rPrPSc can be produced rapidly in high purity, using the rPrP-QuIC method solves the difficulty of using the brain 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. This test can be quantitative and sensitive as *in vivo* testing [56] and has been adapted to different types of TSE. The RT-QuIC assay provides rapid and highly sensitive discrimination of prion-infected and uninfected brain tissues. Furthermore, the technique has proved sensitive in detecting prions in several infected tissues and in fluids such as cerebrospinal fluid, saliva, nasal fluids and blood [59, 60]. This method does not detect prion infectivity in a given tissue, but allow detection of a seeding activity potentially associated with prion replication. Under defined conditions, this method can be used to quantitatively estimate prion concentration in fluids and tissues of interest. Indeed, based upon the quantitative correlation between prion seed concentration and the lag time to the start of the conversion reaction, qRT-QuIC allows quantification of prion infectivity in tissues, body fluids, and excreta [61]. For quantification, the amplified PrPSc signal can be compared with that seen in endpoint titrated material run in the same conditions (such as brain homogenate from animals at the terminal stage of disease) or to PrP calibration curves. By analogy with animal bioassays, RT-QuIC assay can titrate the seeding activity in endpoint diluted samples [56, 59]. Serial dilutions of a given sample are used as seeds and the seeding dose (SD) giving 50% ThT-positive replicate reactions (SD50), that is, the 50% endpoint dilution, is estimated. The SD50 is analogous to the 50% lethal dose (LD50) determined in an endpoint dilution animal bioassay. However, RT-QuIC has several major advantages over animal bioassays, including practicality, high-throughput potential, rapidity, and reduced cost. The quantitative aspect of qRT-QuIC suggests that it can provide a reliable assessment of anti-prion therapy *in vivo* in order to follow the effects of therapy on progression of prion diseases. Moreover, since qRT-QuIC provides an ultra-sensitive method for quantifying pathological amyloid aggregate seeds, this technique may also be applicable to other disease-associated proteins rich in

β-pleated structures that bind T and that 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 to the sensitive detection and discrimination of the C-BSE, L-BSE, and H-BSE (**Figure 10**) [62, 63]. Brain tissue from cattle affected by these strains were tested by the RT-QuIC assay and found that all these forms can be detected and distinguished using particular rPrPSc substrates. RT-QuIC tests have been adapted to the detection of many types of prion seeding activity; however, there are still some missing outcomes for the ante-mortem diagnosis of TSE in animals of farm interest:

- identify the most noninvasive and economic biological peripheral matrix for performing the prion test in living subjects;
- recognize classical and atypical forms of PrPSc by a unique protocol for obtaining a single diagnostic assay for TSE diseases;
- detect PrPSc from all biological fluids by removing soluble components that would inhibit the assay;
- collect pre-clinical and clinical data from subjects resulted PrPSc positive in peripheral matrices to better define the peripheral TSE infectivity distribution.

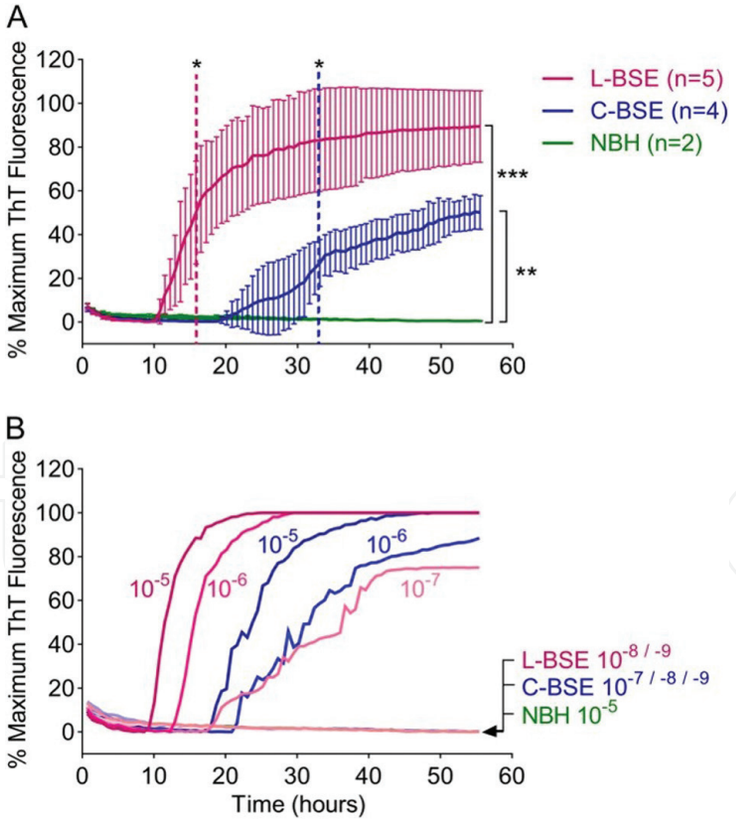


Figure 10. RT-QuIC sensitivity for C-BSE and L-BSE detection. (A) L-BSE-infected (magenta), C-BSE-infected (blue), or normal negative control (NBH, green) 10^{-5} brain tissue dilutions were used to seed quadruplicate RT-QuIC reactions using the Ha-S rPrPSen substrate. (B) Serial dilutions (10^{-5}–10^{-9}) 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 (Orrù et al. 2015).

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