

CHAPTER 2.4.6.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB). It is a transmissible prion disease. The archetype for this group of diseases is scrapie of sheep and goats (see Chapter 2.7.12 Scrapie).

The epizootic of BSE can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat-and-bone meal included in animal feedstuffs. Initial cases of BSE in some countries were considered to be the result of exports from GB of infected cattle or contaminated meat-and-bone meal, although exportations from other countries are now implicated. In others, initial cases have no clear link with imported meat-and-bone meal, suggesting that earlier, undetected indigenous and possibly spontaneous cases may have occurred. As a result of control measures, the epizootics are in decline. Cases of BSE have been detected in most European countries, in North America and in a few Asian countries.

Experimental transmissibility of BSE to cattle has been demonstrated. The BSE agent is also believed to be the common source, via dietary routes, of transmissible spongiform encephalopathies (TSEs) in other ruminant species and felidae. There is evidence of a causal link between the BSE agent and the variant Creutzfeldt-Jakob disease (vCJD) in humans. Recommendations for handling BSE-infected material assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: *Clinical BSE has a peak incidence in cattle aged between 4 and 5 years. The clinical course is variable but can extend to several months. Overt clinical signs are distinctive, if differential diagnoses can be eliminated. Early clinical signs may be subtle and mostly behavioural, and may lead to disposal of affected animals before suspicion of BSE is triggered. In countries with a statutory policy toward the disease, clinically suspect cases must be killed, the brain examined and the carcass destroyed. Now, in most countries, active surveillance at abattoirs identifies infected cattle slaughtered prior to the onset of clinical signs, and the screening of fallen stock identifies cases in which there may have been unrecognised clinical signs. No diagnostic test is currently available for live animals. The nature of the agents causing the TSE is unclear. A disease-specific partially protease-resistant, misfolded isoform of a membrane protein PrP^C, originally designated PrP^{Sc}, has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the principal or sole component of the infectious agent. Confirmation of the diagnosis, formerly by histopathological examination of the brain, is now, therefore, by the application of immunohistochemical (IHC) and/or immunochemical methods to brain tissue for the detection of PrP^{Sc}. PrP^{Sc} can be detected in specific neuroanatomical loci in the CNS of affected cattle by IHC methods in formalin-fixed material, or by immunoblotting and other enzyme immunoassay methods using unfixed brain extracts.*

Transmission from infected brain tissue is the only method currently available for detection of infectivity and is important for characterisation of agent strains. Variant or atypical forms of BSE have been detected across all continents that have experienced classical BSE. While in the majority of instances atypical phenotypes have been based on Western immunoblot banding pattern, bioassay characterisation supports the concept of strain diversity in BSE.

Commercial diagnostic kits for BSE are available and are used for diagnosis of BSE in many countries; similarly a number of anti-PrP antibodies form the basis of many diagnostic methods. Some are available commercially, or from OIE Reference Laboratories or other laboratories with active TSE programmes.

Serological tests: Specific immune responses have not been detected in TSEs.

Requirements for vaccines: There are no vaccines available currently.

A. INTRODUCTION

Bovine spongiform encephalopathy (BSE), a fatal prion disease of cattle, may present clinically with apprehension, hyper-reactivity and ataxia. Without an isolatable causal agent, cases can only be conclusively confirmed post-mortem by the accumulation in the central nervous system (CNS) of prion protein (PrP^{Sc}, PrP^d or PrP^{res}), a partially protease-resistant isoform of a host-encoded protein (PrP^C). The prion hypothesis proposes that the agent is composed entirely of PrP^{Sc}, which is capable of inducing conversion of PrP^C. The function of PrP^C remains unclear. Alternative hypotheses, such as viral or bacterial origins or the involvement of cofactors such as mineral imbalances, remain unproven. The basis for strain variation is still unclear, but within the prion hypothesis, 'strains' are encoded in different conformations of the protein.

Pathological and bioassay characterisation showed that the epidemic was sustained by a single strain, and consistently distinctive neuropathology and PrP^{Sc} molecular profiles in clinically affected animals were the basis for the case definition of BSE. Since 2003, reports of variant pathology (Casalone *et al.*, 2004) and/or molecular characteristics in aged cattle from several countries have indicated possible agent strain variation (for examples, see refs Jacob *et al.*, 2007 and Yamakawa *et al.*, 2003). Most cases were identified through active surveillance of non-suspect populations using rapid PrP immunodetection methods. So far, approximately 50 BSE cases have now been recognised that differ in their molecular profiles by Western immunoblotting from those typically found in the epidemic. Because of the detection of most of these cases by active surveillance, correlation of laboratory diagnostic data with clinical histories is lacking, and most focus only on Western immunoblotting data. An interesting common feature is that most of these variant characteristics originate from older cattle. Initial bioassay data support the hypothesis that these isolates are biologically distinct from classical BSE (Beringue *et al.*, 2006; Lombardi *et al.*, 2008). It is not known whether atypical cases have any relevance to forms of human prion diseases. At present, these atypical cases appear as two distinct types classified by the molecular mass of the unglycosylated PrP^{res} protein band relative to that of classical BSE. One type is of a higher molecular mass (H-type) and the other shows a lower molecular mass (L-type).

Epidemiological studies established that BSE occurred as an extended common source epizootic, through feed-borne exposure (Anderson *et al.*, 1996). BSE occurred in many countries at a lower incidence than in GB. Many cases are most likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal, with subsequent local propagation through contaminated feedstuffs. However, in some countries, cases reflected indigenous exposure (OIE: World animal health situation – Bovine spongiform encephalopathy [http://www.oie.int/eng/info/en_esb.htm]).

There is no evidence of horizontal transmission and little data to support maternal transmission (Prince *et al.*, 2003). Studies have not revealed evidence of risk from semen, milk or embryos (Prince *et al.*, 2003).

The epizootics of BSE have declined, and show the success of controls in the form of changes in age-specific incidence. Interpretation of the status of epizootics has been enhanced by active surveillance detecting infected animals that were not clinically suspect.

The emergence of vCJD in humans has been causally linked to ingestion of BSE (Bruce *et al.*, 1997). Recommended safety precautions for handling the agent are based on the assumption that BSE is zoonotic. Biocontainment for necropsies and tissue handling should be risk-based and compliant with relevant national regulations; any procedure creating aerosols must be conducted under containment level 3 (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities), and the laboratory must comply with national biocontainment and biosafety regulations to protect staff from exposure to the pathogen. Recommended decontamination procedures may not be completely effective when dealing with high-titre material or when the agent is protected within dried organic matter. Recommended physical inactivation is by porous load autoclaving at 134°C–138°C for 18 minutes at 30 lb/in² (208 kPa or 2.2 bar). However, temperatures at the higher end of the range may be less effective than those at the lower end and total inactivation may not be achieved under certain conditions, such as when the test material is in the form of a macerate. Recommended decontamination procedures may not be completely effective under some circumstances. Disinfection of potential fomites is carried out using sodium hypochlorite containing 2% available chlorine, or 2 N sodium hydroxide, applied for more than 1 hour at 20°C for surfaces, or overnight for equipment.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

There is no method capable of confirming the presence of BSE in the live animal. Identification of the 'agent' begins with clinical suspicion of disease, or the post-mortem demonstration of PrP^{Sc} accumulation in a non-suspect animal through active surveillance. The nature of the 'agent' itself remains hypothetical, and it cannot therefore be isolated for diagnostic purposes. However, PrP^{Sc} is widely accepted as a consistent disease marker, and, with the exception of clinical examination and histopathology, all current diagnostic methods are based on the demonstration of this protein.

Clinical 'classical' BSE occurs in adult cattle (ranging between 20 months and 22 years in the UK). During the main epidemic most cases were observed in dairy cattle aged 4–6 years. Subsequently the impact of effective controls has been reflected in an increasing age at onset of clinical disease. BSE has an insidious onset and usually a slowly progressive course (Konold *et al.*, 2004; Wilesmith *et al.*, 1992). The onset of clinical signs is not associated with season or stage of breeding cycle. Occasionally, a case will present with acute signs and then deteriorate rapidly, although frequency of observation is a significant factor in determining early clinical signs. Presenting signs, though variable, usually include changes in behaviour and temperament, hyper-reactivity and incoordination. Affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking, which is often the first observed sign. In dry cows especially, hind-limb incoordination and weakness can be the first clinical features to be noticed. The most commonly reported nervous signs have been apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. Startle responses to external stimuli, which are repeatable, are frequent and thus used to support the clinical diagnosis in suspect BSE cases (Konold *et al.*, 2004). The intense pruritus characteristic of most sheep with scrapie is not prominent in cattle with BSE. Affected cows will sometimes stand with low head carriage and the neck extended, an arched back or wide-based hind limbs. A tremor of the head may also be visible. Abnormalities of gait, such as incoordination and hypermetria, are usually confined to the hind limbs and are most readily appreciated when cattle are observed at pasture. With advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. General clinical features of loss of bodily condition and reduction in milk yield often accompany nervous signs as the disease progresses. There has been no change in the clinical picture of BSE over the course of the epizootic in the UK (Konold *et al.*, 2004; Wilesmith *et al.*, 1992). Clinical signs are essentially similar in other countries where BSE has occurred. The protracted clinical course, extending usually over a period of weeks or months, would eventually require slaughter on welfare considerations. However, a statutory policy to determine the BSE status of a country requires compulsory notification and diagnostic investigation of clinically suspect cases, their slaughter and post-mortem examination of the brain. Early in the disease course, the signs may be subtle, variable and nonspecific, and thus may prevent clinical diagnosis on an initial examination. Continued observation of such equivocal cases, together with appropriate clinical pathology procedures to eliminate differential diagnoses, especially metabolic disorders, will establish the essential progression of signs. Some early clinical signs of BSE may show similarities with features of nervous ketosis, hypomagnesaemia, encephalic listeriosis and other central nervous system diseases. Subtle signs may sometimes be exacerbated following stress, such as that caused by transport. Video clips of cattle affected by BSE may be downloaded from the web site of the OIE Reference Laboratory for BSE in the UK¹, which also provides DVD or videotape footage upon request, or the OIE Reference Laboratory in Switzerland².

The small number of variant forms of TSE in cattle, operationally defined as BASE (bovine amyloidotic spongiform encephalopathy) or L-type and H-type BSE based on the mass of the unglycosylated PrP fragment in Western blots (Casalone *et al.*, 2004; Jacob *et al.*, 2007) have yielded little clinical information as most have been identified in apparently healthy cattle or fallen stock.

The laboratory diagnosis of BSE has evolved with increasing knowledge of the disease pathogenesis and technical advances (Gavier-Widen *et al.*, 2005). In the absence of *in-vitro* methods for isolation of the causative agent, the historical basis of disease confirmation was the demonstration of the morphological features of spongiform encephalopathy by histopathological examination, at several different levels of the brain. Histopathology remains the only method by which this characteristic TSE-specific vacuolation can be identified. The original diagnosis of BSE was based on the identification of histopathological features consistent with a scrapie-like spongiform encephalopathy. This was supported by the electron microscopic visualisation of fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrP^{Sc}, in detergent extracts of affected brain. The material examined was invariably from suspect clinical cases. During the rapidly increasing epizootic in the late 1980s, histopathological diagnosis based on examination of a single section of medulla oblongata (at the level of the obex) was validated against more extensive examination of the brainstem. This simplified approach enabled modification of sampling of the fresh brain; instead of whole brain removal, the required section was

1 The laboratory is also the European Commission (EC) Transmissible Spongiform Encephalopathies (TSEs) Community Reference Laboratory. All its Web Resources can be found at: http://www.vla.gov.uk/science/sci_tse_rl_tests.htm
2 www.neurocenter-bern.ch

taken from brainstem removed via the *foramen magnum*, using customised instrumentation. As the diagnostic specificity of PrP^{Sc} was established, immunochemical methods of disease-specific PrP detection, including IHC techniques and Western blotting/SAF-immunoblotting, were used, in addition to histopathology, to confirm the diagnosis and improve diagnostic sensitivity in early or autolysed cases. The introduction of more rapidly performed *in-vitro* methods, mostly enzyme-linked immunosorbent assay (ELISA)-based, for the detection of PrP^{Sc} has led to a variety of 'rapid', tests, which are now the principal screening tools for active surveillance. Such tests provide a preliminary diagnosis from which positive or inconclusive results are subject to examinations by IHC or Western blot confirmatory methods. Rapid tests are currently the main approach by which cases are detected and their wider use as part of the confirmatory process has been agreed (OIE Reference Laboratory in the UK). All currently recognised forms of BSE are detectable by these methods although a full evaluation for atypical forms (H and L types) has not been carried out.

The choice of any particular method will depend on the context of its use. Contexts will extend from confirmation of clinical suspects to the screening of healthy populations for evidence of covert or preclinical disease. The case definition adopted will also differ according to whether the diagnostic method is to be applied for confirmation or for screening. Care should be taken in the interpretation of diagnostic data using methodologies that do not enable careful cross-referencing with the standards for confirmatory diagnosis that are defined here. Without appropriate comparison with previously published criteria defining the BSE phenotype, and in the absence of transmission studies, diagnostic results that claim the identification of a new strain may be premature. Quality control (QC) and quality assurance (QA) are essential parts of the testing procedures and advice can be supplied by the OIE Reference Laboratories (http://www.oie.int/eng/OIE/organisation/en_LR.htm). Whether BSE-infected animals are identified by passive or active surveillance, it is good practice to detect and confirm disease by a combination of at least two test methods. The primary test can be one of the confirmatory test methods described below or a rapid test, but it is important to apply a secondary test to confirm a positive or inconclusive primary test result. Where there is a conflict between primary and secondary test results, further tests using immunohistochemistry or scrapie-associated fibrils (SAF)-immunoblot (or approved alternative) should be applied or samples should be submitted to an OIE Reference Laboratory for resolution.

a) Sample preparation

The BSE status of a country, the relative implementation of passive and active surveillance programmes and the diagnostic methods applied, will all influence sampling strategy.

In all circumstances of passive surveillance of neurological disease in adult cattle **where the occurrence of BSE within a country or state has not been established or is of low incidence**, it is recommended that clinically suspect cases are subjected to a standard neuropathological approach in which the whole brain is sampled, and a range of representative areas examined. Cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution preceded, if necessary, by sedation. The brain should be removed as soon as possible after death by standard methods. There are no gross lesions associated with BSE, so if any are observed when the brain is removed, these should be specifically sampled to facilitate differential diagnosis.

Care must be taken to preserve suitable fixed and fresh brain samples for the immunohistochemical and immunochemical detection of PrP^{Sc}. Departure from this approach of collecting and retaining the entire brain may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE.

Histopathology and IHC examinations are carried out initially on a single block (0.5–1.0 cm in width) cut at the obex of the medulla oblongata (Fig. 1a and b, level A–A representing the centre of the block for examination), which should be fixed for a minimum of 3–5 days (dependent on block thickness) in 4% formaldehyde solution (i.e. 10% formal saline or 10% normal buffered formalin [NBF]). Subsequent histological processing should be by conventional paraffin wax embedding methods for neural tissue. (An example of appropriate processing methods can be obtained from the OIE Reference Laboratory in the UK.

Fresh material for the immuno-detection of PrP^{Sc} should be taken initially as a hemisection of the medulla to the level of the obex, or as a complete coronal section (2–4 g), immediately rostral, or caudal, to the obex block taken for fixation. All other brain areas should be subdivided by a saggital paramedian cut (3–5 mm off the median). The smaller portion is reserved for the PrP^{Sc} detection by immunochemical methods (e.g. immunoblot) and is stored frozen prior to testing (if testing is not done immediately after sampling). After sampling of the obex region for fixation and sampling of fresh tissue, the larger portion of the brain tissue is placed, intact, in approximately 4–6 litres of 10% formalin fixative, which should be changed twice weekly. After fixation for 2 weeks, if further investigation is necessary, the brain can be cut into coronal slices. The fixation time may be shortened by cutting the fresh brainstem (detached from the rest of the brain) into smaller coronal pieces, similarly to the initial removal of the obex region, but leaving intact the remaining diagnostically important cross-sectional areas at the levels of the cerebellar peduncles and the rostral colliculi (Figure 1a and b, levels B–B and C–C, respectively). Depending on some other factors (temperature,

agitation, thickness of tissue block, use of microwave) the fixation time for these smaller pieces of brain may be reduced. However, evaluation of the effects of these kinds of accelerated fixation processes on subsequent IHC protocols needs to satisfy proficiency testing standards. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks' fixation.

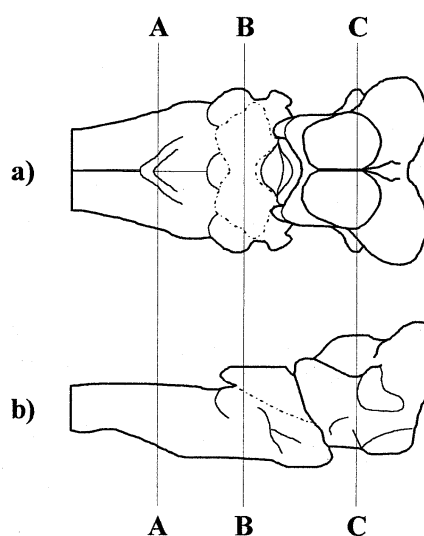


Fig 1. Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects.

Recommended levels at which sections should be taken:

A–A = medulla, at the obex; B–B = medulla through caudal cerebellar peduncles;

C–C = midbrain through rostral colliculi.

When the occurrence of BSE in a particular country has been established in the indigenous cattle population, and there is evidence that the distribution of lesions, and other aspects of disease phenotype are consistent with those described for classical BSE, it is adequate, although not ideal, for disease confirmation and monitoring purposes, to remove the brainstem alone.

This can be achieved via the *foramen magnum* without removal of the calvarium (Fig. 2). This will reduce the amount of fixative required and the time and equipment needed, thereby lowering costs and improving safety. The major target areas for histological examination can still be maintained. This method allows for collecting and examining a large number of samples for passive surveillance or for an active surveillance programme in abattoirs. The brainstem is dissected through the *foramen magnum* without opening the skull by means of a specially designed spoon-shaped instrument with sharp edges around the shallow bowl. Such instruments are available commercially. It is possible that variations in technique, including orientation, are required with different forms of the instrument, and it is important to train operators once there is agreement on equipment to be used. This training should include information on the cross-sectional distribution of PrP^{Sc} in the brainstem, and the need for the accurate sampling and preservation of the diagnostic target areas (see below). Under abattoir conditions it has also been shown possible to obtain expulsion of intact brainstem via the *foramen magnum*, providing histologically good material, by application of pressure (air or water) (Hejazi & Danyluk, 2005) through the entry wound in the skull when penetrative stunning has been used in slaughtering. Clearly the feasibility and efficacy of this method will be dependent on the slaughter method and before implementation for routine use requires local risk assessment.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation are unlikely to be available. In most countries, brainstem alone is collected, even before the first confirmation of BSE. Provision should be made for heads that have been sampled in the course of active surveillance to be retained until the outcome of initial testing is available. This would enable comprehensive sampling of the brain of positive animals in retrospect for the characterisation of cases. This is particularly important if in-house tests that are not subject to external quality assurance are used and where, in the absence of direct comparison with the methods described here, claims are made that new phenotypes have been identified. Where rapid immunoassays are used as the primary surveillance tool it is necessary to make material available for further morphological (including immunohistochemistry) and molecular examination that would allow identification of disease phenotype in the absence of a diagnosis of BSE having ever been made in that country.

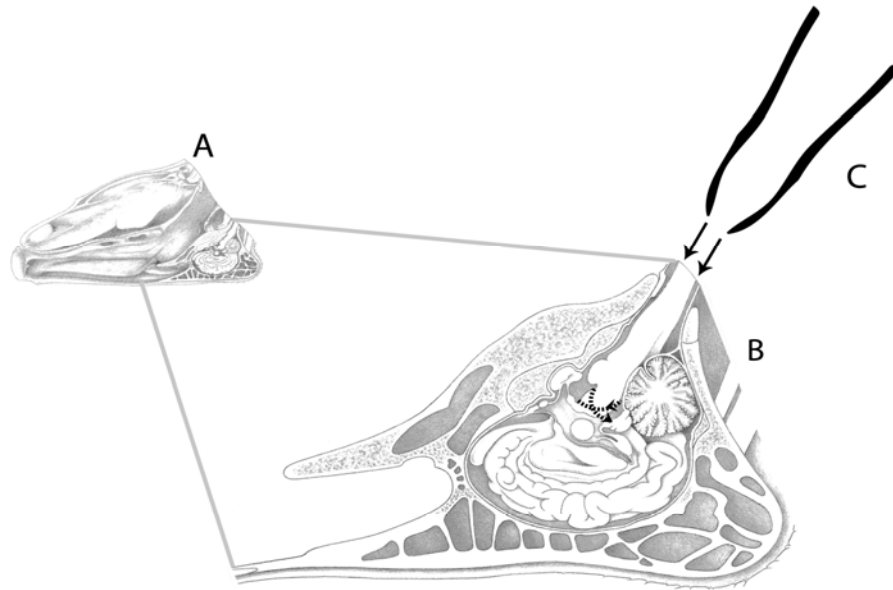


Fig. 2. After the head has been removed from the body by cutting between the atlas vertebra and the occipital condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing of cranium). The instrument (C) is inserted through the foramen magnum between the dura mater and the ventral/dorsal aspect (depending upon the specific approach) of the medulla and advanced rostrally, keeping the convexity of the bowl of the instrument applied to the bone of the skull and moving with a side-to-side rotational action. This severs the cranial nerve roots without damaging the brain tissue. The instrument is passed rostrally for approximately 7 cm in this way and then angled sharply (i.e. toward the dorsal/ventral aspect of the brainstem, depending on the approach) to cut and separate the brainstem (with some fragments of cerebellum) from the rest of the brain. The instrument, kept in the angled position, is then withdrawn from the skull to eject the tissue through the foramen magnum.

- **Sampling of brainstem in active surveillance with use of rapid tests**

The sampling and processing of brain tissue for use in any rapid test should be carried out precisely as specified by the supplier or manufacturer of the test method or kit. Details of this procedure vary from method to method and should not be changed without supportive validation data from the manufacturer for the variant methodology. The preferred sample for immunoassay should be at, or within 1.0 cm rostral, or caudal to, the obex, based on the caudo-rostral extent of the key target sites (Fig. 3) for demonstration of PrP^{Sc} accumulations and the evaluation of sampling for rapid tests. The choice of target site has to take into account the subsequent method of confirmation. At least a hemi-section of the medulla at the level of the obex should be kept intact for fixation for immunohistochemistry/histology (as described above) should a positive result require confirmation. Sampling the medulla rostral or caudal to the obex for rapid testing does not compromise examination by histological or IHC means. However, to obtain comparable samples for rapid *and* confirmatory testing, sampling by hemi-section of the medulla at the level of the obex is preferable. While there is resultant loss of the ability to assess the symmetry of any histopathological lesions (notably vacuolation), this approach is less likely to compromise the more important IHC examination. If hemisectioning is adopted however, it becomes critical to ensure that the target sites are not compromised in either sample. For example, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (target areas for lesions in cattle with BSE) are small, and lie relatively close to the midline (Fig. 3). If sampled tissue is autolysed to the point that anatomical orientation is not possible, an unidentified aliquot can still be taken and tested. A positive result in such cases is still a valid result, but a negative test result cannot be taken to indicate a negative animal, and it should be interpreted with caution and reported with appropriate qualification.

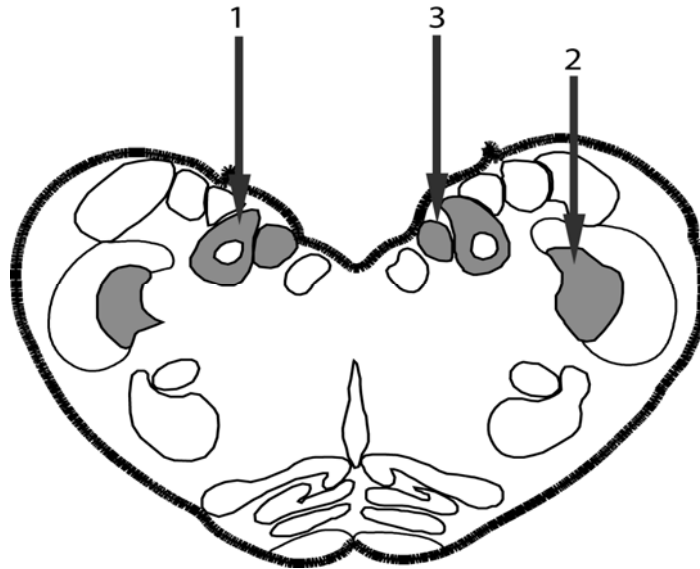


Fig 3. Cross section of the bovine brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE. These are principally the nucleus of the solitary tract [1] and the nucleus of the spinal tract of the trigeminal nerve [2]; but also the dorsal motor nucleus of the vagus nerve [3]. It follows that material taken for application of a rapid test must also include representation of these areas.

Inaccurate hemi-sectioning could result in the complete loss of a target area for confirmatory testing, and could compromise a surveillance programme. Failure to accurately sample target areas may also arise through inappropriate placement of proprietary sampling tools. Such approaches therefore need to be implemented with a very clear policy and monitoring programme for training and quality assurance of sampling procedures, including anatomical positioning, and not just sample weight. Because of the specifically targeted distribution of PrP^{Sc}, sample size and location should be as described in the diagnostic kit or, if not specified, at least 0.5 g taken from the diagnostic target areas for all confirmatory tests as detailed in Fig 3. Performance characteristics of the tests may be compromised by autolysis, particularly due to loss of the ability to ensure inclusion of target areas in the sample taken from the diagnostic target areas detailed in Fig 3.

b) Diagnostic examination

i) Histological examination

Histopathology is no longer the diagnostic method of choice for investigation of suspect animals, or the screening of healthy populations. However, an awareness of the histopathological changes is important, to facilitate detection of cases when conducting routine diagnostic histological examinations of cattle brains sampled for reasons other than BSE testing. For differential diagnosis, sections of medulla–obex are cut at 5 µm thickness and stained with haematoxylin and eosin (H&E). If tissue quality permits, the histopathological examination of H&E sections allows confirmation of the characteristic neuropathological changes of BSE (Simmons *et al.*, 1996; Wells & Wilesmith, 1995) by which the disease was first detected as a spongiform encephalopathy. These changes comprise mainly spongiform change and neuronal vacuolation and are closely similar to those of all other animal TSEs, but in BSE the frequent occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex can provide a satisfactory means of establishing a histopathological diagnosis on a single section of the medulla. As in other species, vacuolar changes in the brains of cattle, particularly vacuoles within neuronal perikarya of the red and oculomotor nuclei of the midbrain are an incidental finding (Gavier-Widen *et al.*, 2001). The histopathological diagnosis of BSE must therefore not rely on the presence of vacuolated neurons alone, particularly in these anatomical locations.

Irrespective of the histopathological diagnosis, immunohistochemistry is routinely employed in addition, as unpublished evidence suggests that as many as 5% of clinical suspects (which are negative on H&E section examination for vacuolar changes at the obex) can be diagnosed by IHC examination. Clearly, examination of the medulla–obex does not allow a full neuropathological examination for differential diagnoses, nor does it allow a comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended to remove whole brains from all clinical suspects. There are still insufficient data available to describe specific histopathological features of H or L type BSE. There are some histological data from Italian researchers on BASE (L-type BSE) (Casalone *et al.*, 2004). Few atypical BSE cases have been found in passive surveillance and it is not possible to obtain whole brains in active surveillance to increase our

knowledge in this respect. The poor condition of the brain of fallen stock, where most atypical cases have been identified, also rules out a complete histological examination because of the effects of autolysis. Experimental transmission studies of L- and H-type BSE into calves and adults are underway in some countries and this should enable histological comparisons with the accumulated data already obtained for classical BSE.

ii) *Detection of disease-specific forms of PrP*

The universal use of PrP detection methods provides a disease specific means of diagnosis independent of the morphological changes defined by histopathology. Many laboratories now supplement or have replaced histopathological examination by IHC and/or other PrP-detection methods. The detection of accumulations of PrP^{Sc} is the approach of choice for surveillance programmes and confirmatory diagnosis. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (Debeer *et al.*, 2002). Freezing prior to fixation will not compromise the immunoreactivity of a sample, but it may compromise the proper identification of target sites. A positive case will have disease-specific immunolabelling (Casalone *et al.*, 2006) in at least one of the diagnostic target areas. For a case to be diagnosed as negative it must be possible to identify the presence of the target areas and to demonstrate that the IHC 'run' was technically successful through appropriate controls. If there is no disease-specific immunolabelling, and target areas cannot be identified, the case should be classified as 'unconfirmed' as opposed to negative. Both H and L-type variants demonstrate accumulation of PrP^{Sc} in the medulla at the level of the obex (Casalone *et al.*, 2006; Gavier-Widen *et al.*, 2008; and the OIE Reference Laboratory in the UK). The range and morphological appearance of immunolabelling throughout the neuraxis differ, particularly in L-type, where PrP^{Sc} is more abundant in the frontal cortical areas, and tends to occur as multiple small plaque-like deposits.

- **Immunohistochemical (IHC) methods**

IHC examination for PrP^{Sc} accumulation is performed on the same formalin-fixed paraffin-embedded material used for the histopathological diagnosis. Different protocols have been applied successfully to the IHC detection of PrP^{Sc} for the diagnosis of BSE and although a standardised IHC method would seem desirable, it might be more important to recognise robust methods that achieve a standardised output, as monitored by participation in proficiency testing exercises, and by comparison with the results of a standardised model method in a Reference Laboratory. The generic technique established for histopathology still applies and it works well in autolysed tissues in which morphological evaluation is no longer possible (Monleon *et al.*, 2003). However, it is imperative to recognise the anatomy of the sample to determine whether or not target areas are represented. This is essential for a negative diagnosis, and may also be pivotal in accurately interpreting equivocal immunolabelling. IHC detection of PrP^{Sc} accumulations approximates to the sensitivity of the Western blotting approach for detection of PrP^{Sc} (Schaller *et al.*, 1999). In combination with good histological preparations, immunohistochemistry allows detection of PrP^{Sc} accumulations and, as this, like the vacuolar pathology, exhibits a typical distribution pattern and appearance. This provides simultaneous evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by reference to the OIE Reference Laboratories.

In contrast to the diagnosis of scrapie of sheep, the limited detection of PrP^{Sc} in lymphoid tissues in BSE does not provide any scope for utilising such tissues for preclinical diagnosis by biopsy techniques.

- **Western blot methods**

Immunoblotting techniques, are carried out on fresh (unfixed) tissue, and can be applied successfully even when tissue is autolysed (Hayashi *et al.*, 2004). The SAF-immunoblot (OIE Reference Laboratory in the UK) 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 immunohistochemistry, for the confirmation of BSE. It is a highly sensitive method using a large mass (ideally 2–4 g) of starting material and several steps to concentrate PrP^{Sc}. Alternative less time-consuming and less costly methods are now available. These use less material and are more practical, but in cases where the amount of PrP^{Sc} is at very low concentrations, they can be less sensitive. Most of these techniques use a precipitation of PrP^{Sc} using phosphotungstic acid (PTA) or other chemicals, and some are commercially available. A range of Western immunoblotting methods are available on the website of the OIE Reference Laboratory in the UK or from the other OIE BSE Reference Laboratories.

While Western blot methodology is now in general use around the world, analytical sensitivity when used to detect PrP^{Sc} varies significantly between methods and laboratories. Where in-house methods are preferred to published methods for confirmatory purposes, it is important that they are evaluated as being fit for purpose and validated in consultation with an OIE Reference Laboratory.

- **Rapid test methods**

Rapid Western blot, lateral flow device (LFD) and ELISA techniques have been developed that allow screening of large numbers of brain samples and are commercially available. Such techniques can be performed in a few hours (see EC evaluations of rapid tests for the detection of BSE on known IHC positive and negative sample groups [OIE Reference Laboratory in the UK]). Tests that have been evaluated and approved for BSE surveillance within the EU are listed in Annex C Chapter X of the TSE Regulation (EC 999/2001 and subsequent amendments). An algorithm of how these tests may be used is available on the website of the OIE Reference Laboratory in the UK.

While many countries, and an OIE *ad hoc* Group on BSE tests, accept EU approval as an indicator of test performance, others have established their own evaluation mechanisms, most notably the United States of America, Canada and Japan (OIE Reference Laboratory in the UK; Canada's protocols for BSE surveillance³; National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, Outline of Regulation System of Veterinary Drugs in Japan⁴). The OIE also has an approval process and protocols for such evaluations are posted on the OIE web site: Validation and certification of Diagnostic Assays⁵), and the EU approval process has been accepted as the gold standard for future evaluations in terms of acceptable sensitivity and specificity.

The relative sensitivities of rapid tests, immunohistochemistry and other confirmatory methods have not been fully determined as, by definition, the tests cannot all be applied to identical samples, and the PrP^{Sc} distribution is anatomically variable. (As a compromise, tissue homogenates or mixtures of finely chopped tissue may be used and provide some information, for certain types of tests.) Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE and are likely to have lower depositions of PrP^{Sc}, than cattle with more advanced disease. Initial evaluations completed in the EU were restricted to a comparison of the examination of a sample of brains of cattle identified as suspect clinical animals with histopathological changes characteristic of BSE and a sample of brains of cattle from New Zealand that were unexposed to BSE and histopathologically negative. Later studies have involved field trial evaluation of potential new tests compared with methods that are already approved. Any discrepant results were resolved by a variety of confirmatory approaches in an OIE Reference Laboratory. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period (Arnold *et al.*, 2007), for example in surveys of post-mortem material collected from routinely slaughtered cattle. In countries conducting surveillance for the detection of the novel occurrence of BSE and in those countries in which a means, independent of the system of notification of suspect cases, of assessing the prevalence of BSE is considered necessary, these screening tests offer an efficient approach. Since their introduction for active screening in Europe from January 2001, such tests have been responsible for the identification of the majority of BSE-infected animals and are the preferred primary test. However, confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by immunohistochemistry or the application of an appropriate Western blot protocol. In 2006, the OIE accepted that through their use in active surveillance programmes, commercial rapid tests have proved themselves to be very effective and consistent, provided they are performed by appropriately trained personnel. Indeed, at times they may out-perform the acknowledged standard of comparison if training and experience in the latter are deficient. Under such circumstances, it is now considered acceptable for diagnosis, even if not ideal for characterisation, for rapid tests to be used in combination for both primary screening in active or passive surveillance programmes and subsequent confirmation. It is essential however to ensure that the choice of primary and secondary test are compatible, and do not present a danger of generating false positive results through shared reagents. Consequently, an algorithm of preferred test combinations will be maintained on the website of the OIE Reference Laboratory in the UK to assist those who wish to use this approach instead of histopathology and immunohistochemistry, or SAF immunoblot for confirmation. The combination of tests should include a Western blot method to generate useful complementary data that will assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue. The confirmation should be carried out in a National Reference Laboratory.

The combination of the two rapid tests can only be used for the confirmation of a BSE case. A negative result by the secondary test is insufficient to define a case as negative following a primary positive result. BSE suspect cases with discordant rapid test results must therefore be investigated further using either the SAF-immunoblot (or approved alternative) or IHC for the demonstration of PrP^{Sc}, or if these methods are not available, by histopathology. If histopathology is unable to confirm the initial reactive result, samples should be submitted to an OIE Reference laboratory for further examination.

Although the test evaluation programmes conducted in Europe were in support of legislation on surveillance for BSE, the consequences are of relevance to other countries. The consequences of false-positive or false-

3 <http://www.inspection.gc.ca/english/anima/disemala/bseesb/surv/protoce.shtml>
4 <http://www.maff.go.jp/nval/english/pdf/outline080514.pdf>
5 http://www.oie.int/vcda/eng/en_background_vcda.htm

negative results are so great that the introduction of new tests should be supported by thorough evaluation of test performance. Claims by test manufacturers should always be supported by data, ideally evaluated independently. It must be stressed that the process of full validation of all of these diagnostic methods for BSE has been restrained by the lack of a true gold standard and the consequent need to apply standards of comparison based on relatively small studies. There is therefore a continuing need for the publication of larger scale studies of assay performance, and none of the data published so far equate with recognised procedures for test validation for other diseases.

d) Other diagnostic tests

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.7.12 Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue has been used as an additional diagnostic method for BSE and has been particularly useful when histopathological approaches were precluded by the occurrence of post-mortem decomposition. With modification, the method may be applied successfully to formalin-fixed tissue (Chaplin *et al.*, 1998). Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE, but does not offer the sensitivity available from IHC or immunoblotting methods. BSE infectivity can be shown by inoculating mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. It is, however, the nearest approach to a 'gold standard' for the characterisation of isolates, which has to be based on secondary biological properties in a standardised host, in the absence of an isolatable physical agent. Transgenic mice, such as those over-expressing the bovine PrP gene, offer bioassays with reduced incubation periods for BSE, but none as yet represent practical diagnostic tools.

There remains the need for a live animal test for BSE with a sensitivity capable of detecting PrP^{Sc} at low levels early in the incubation of the disease. As yet, the effectiveness of potential approaches has not been shown. The EC remains committed to the evaluation of *in-vivo* tests, and sets out protocols for the evaluation of such tests (European Food Safety Authority (EFSA). Opinions of the Scientific Panel on Biological Hazards (http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620776473.htm). The detection of certain protein markers of neurodegeneration, including apolipoprotein E (Apo E), the 14-3-3 protein and S-100 proteins in cerebrospinal fluid have not proved useful for diagnosis of preclinical cases of BSE. The diagnostic potential of the observation of IgG light chains as a surrogate marker for prion infection in the urine of scrapie-infected hamsters, has not been investigated for the diagnosis of BSE.

e) Availability of diagnostic reagents and kits

As discussed previously, diagnostic kits have been licensed for use in many countries and reagents are available commercially and from OIE reference and other laboratories with a TSE programme.

2. Serological tests

The infectious agents of prion diseases cannot easily be grown *in vitro* and do not induce a significant immune response in the host.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available currently.

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NB: There are OIE Reference Laboratories for Bovine spongiform encephalopathy (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).