

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease of adult cattle that was first recognised in Great Britain (GB) in 1986. It is a transmissible spongiform encephalopathy or 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 proprietary concentrates or feed supplements. 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 are clearly indigenous, with no clear link with imported meat-and-bone meal, suggesting that earlier, undetected, cases may have occurred. As a result of control measures, the epizootics in many countries are in decline. Cases of BSE currently occur throughout most of Europe and have been detected in Asia and North America.

Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposures to brain tissue from affected cattle. The BSE agent is also believed to be the common source, via dietary routes, of transmissible spongiform encephalopathies (TSEs) in some other ruminant species and in species of felidae. There is evidence of a causal link between the BSE agent and the variant form of the human TSE, Creutzfeldt-Jakob disease (vCJD). Recommendations for safety precautions for handling BSE-infected material now assume that BSE is a zoonosis and a containment category 3 (with derogation) has been ascribed.

Identification of the agent: *In GB, BSE had 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 sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are 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 identifies infected cattle before, or without, the recognition of clinical signs. No diagnostic test for the BSE agent in the live animal is presently available. 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, usually to conventional or transgenic mice, is the only practical method currently available for detection of infectivity and has an important role in the confirmation or 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 of some isolates provides emerging evidence of strain diversity in naturally occurring prion diseases of cattle.

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

Requirements for vaccines and diagnostic biologicals: *There are no biological products available currently. Commercial diagnostic kits for BSE are available and are used for diagnosis of BSE in many countries.*

A. INTRODUCTION

BSE is a fatal disease of domestic cattle, cases of which were first recognised in Great Britain (GB) in November 1986 (27, 37). It is a transmissible spongiform encephalopathy (TSE) or prion disease, originally typified in animal species by scrapie of sheep. Prion diseases are defined by the pathological accumulation, principally and consistently in the central nervous system (CNS) and more variably in the lymphoreticular system (LRS), of a misfolded, partially protease-resistant, isoform of a highly conserved, host-encoded membrane protein (PrP^C), which was originally designated PrP^{Sc}. The function of PrP^C remains unclear. PrP^{Sc} is the only disease-specific macromolecule identified in the scrapie-like diseases. It is also variably referred to as PrP^{res}, to denote the proteinase resistant property of the pathological protein, PrP^d for disease-specific and PrP^{bse} specifically in BSE. Here PrP^{Sc} is used generically to refer to the abnormal isoform of PrP^C. The favoured scientific view is that the agent is composed entirely of the disease-specific isoform of PrP and that the altered form is capable of inducing conversion of the normal form: the protein only or 'prion' hypothesis. Data in support of alternative hypotheses, such as viral or bacterial origins or the involvement of cofactors such as mineral imbalances, remain elusive. The molecular basis for strain variation is still unclear, but according to the prion hypothesis strain characteristics are encoded in different conformations of the prion protein.

Initial characterisation of BSE isolates from GB by transmission to mice showed that over the main course of the epidemic the disease was caused by a single major strain of agent that differed from characterised strains of the scrapie agent in sheep (4). Uniformity of the pathology among most affected cattle has also supported the notion of a consistent disease phenotype for BSE (7, 30). The pattern of neuropathology in the host species is important in the phenotypic characterisation and consequent case definition of BSE used for confirmation of the disease. Reports since 2003 of variant features of pathology and/or molecular characteristics in several countries have raised issues of possible agent strain variations of prion disease in cattle (3, 8, 21, 44). Whether or not such findings represent true strain variation of the BSE agent, or different forms of prion infections of bovines, remains to be proven. Because of the detection of most of these cases by active surveillance, correlation with clinical histories is lacking, and most focus only on western immunoblotting data (3, 44). The most comprehensive description, providing immunohistochemical (IHC), histopathological and western immunoblotting characterisation relates to two aged cows in Italy (8). Transmissibility of certain isolates to mice, with features distinct from previous BSE transmissions has been confirmed (2, 5). Transmission studies of other isolates in cattle are in progress. An interesting common feature is that most of these isolates originate from older cattle.

The initial epidemiological studies of BSE in GB established that its occurrence was in the form of an extended common source epizootic, due to feed-borne infection with a scrapie-like agent in meat-and-bone meal used as a dietary protein supplement (1, 39). Although recorded initially in the United Kingdom (UK), BSE has now occurred, albeit at lower incidence, in many countries involving imported and/or indigenous cattle. Such cases are most likely to have resulted directly or indirectly from the export of infected cattle or infected meat-and-bone meal from countries with occurrences of BSE, including historically, the UK. It is clear that infection has subsequently been propagated within countries in which cases have occurred as highlighted by the evaluation of Geographical BSE Risk (GBR) in many countries by the Scientific Steering Committee of the European Union (13). Indeed, in some countries, the only cases detected reflect indigenous exposure rather than direct linkage with imported contaminated feed (41). Current statistics on BSE occurrence around the world are provided by the OIE (41).

There is no evidence of horizontal transmission of BSE between cattle and little data to support the existence of maternal transmission (27). Epidemiological and transmission studies have not revealed evidence of a risk from semen or milk or through embryos (27).

As a result of control measures, the epizootics in the UK and many other countries have declined, or show the effects of controls in the form of changes in age-specific incidence. In some countries the controls have not been in place long enough for the effects to be recognised. Interpretation of the status of epizootics has been enhanced by the introduction of active surveillance using rapid diagnostic tests, which have detected infected animals that have not been recognised as clinically suspect cases. While such active surveillance is capable of detecting a proportion of preclinical cases, retrospective investigation at farms of origin frequently confirms that some signs have been presented before slaughter, but had not triggered consideration of a clinical diagnosis of BSE.

The novel occurrence of TSEs in several species of captive exotic bovidae and felidae and in domestic cats during the course of the BSE epizootic is attributed to and, for several affected species, shown, to have been caused by the BSE agent (23). Exposure is presumed to have been dietary.

The emergence of a new form of the human prion disorder Creutzfeldt-Jakob disease (CJD), termed variant CJD (vCJD) in the UK (40) has also been shown by transmission and molecular studies (6, 10) to be causally linked to the BSE agent. Dietary exposure is considered the route of infection. In the past, no connection has been established between the exposure of humans to agents causing animal spongiform encephalopathies and the occurrence of the human TSE and thus BSE presents a precedent as a zoonotic TSE. It is therefore now recommended that safety precautions for handling the BSE agent be based on the assumption that BSE is transmissible to humans. The epizootic of vCJD in the UK in individuals homozygous for MM at codon 129 of the PrP gene, peaked in 2000; small numbers of cases have occurred in some other countries.

Consequent to the occurrence of vCJD, a risk-based approach should be adopted when determining the biocontainment level for conducting necropsies on BSE-suspect animals or handling tissues derived from such animals, but any procedure that creates 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. 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. Disinfection 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 (33).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Clinical BSE, as it presented throughout the main epidemic, occurs in adult cattle, and most cases were observed in dairy cattle aged 4–5 years. With the decline of the epidemic, the impact of effective controls has been reflected in an increasing age at onset of clinical disease (27). Onset of clinical signs is not associated with season or stage of breeding cycle. BSE has an insidious onset and usually a slowly progressive course (24, 38). 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 behavioural changes, apprehension, and hyper-reactivity. For example, affected cows may be reluctant to enter the milking parlour or may kick vigorously during milking. In dry cows especially, hind-limb incoordination and weakness can be the first clinical features to be noticed. Neurological signs predominate throughout the clinical course and may include many aspects of altered mental status, abnormalities of posture and movement, and aberrant sensation, but the most commonly reported nervous signs have been apprehension, pelvic limb ataxia, and hyperaesthesia to touch and sound. The intense pruritus characteristic of some sheep with scrapie is not prominent in cattle with BSE, though in a proportion of cases there is rubbing and scratching activity. Affected cows will sometimes stand with low head carriage, the neck extended and the ears directed caudally. Abnormalities of gait include swaying of the pelvic quarters and pelvic limb hypermetria; features that are most readily appreciated when cattle are observed at pasture. Gait ataxia may also involve the forelimbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. Reports of reduced rumination, also bradycardia and altered heart rhythm, though not specific signs, suggest that autonomic disturbance is a feature of BSE. General clinical features of loss of bodily condition, decreasing live weight, 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 (24, 38). 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 encephalitides. 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 European Commission (EC) TSE Community Reference Laboratory/Veterinary Laboratories Agency (VLA) (15). DVD or videotape footage of the clinical signs is available from this and other sources (35).

The laboratory diagnosis of BSE has evolved in concert with increasing knowledge of the disease and technical advances (17). In the absence of *in-vitro* methods for isolation of the causative agent, the historical basis of confirmation of the diagnosis in this group of diseases was the demonstration of the morphological features of spongiform encephalopathy by histopathological examination. This remains necessarily, by definition, the only method by which this characteristic vacuolar pathology can be diagnosed. The original diagnosis of BSE was

based on the histopathological features of a scrapie-like spongiform encephalopathy and 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. In GB, in the light of the rapidly increasing epizootic in the late 1980s, histopathological diagnosis based on examination of a single section of medulla oblongata taken at the level of the obex, was validated against more extensive examination of the brainstem (34). This simplified approach enabled modification of sampling of the fresh brain; instead of whole brain removal, the required section was taken from the brainstem removed via the *foramen magnum*, using customised instrumentation. With increasing recognition of the diagnostic specificity of PrP^{Sc} and, with availability of appropriate antibodies and increasing efficiency of detection methods, 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. The introduction of more rapidly performed *in-vitro* methods for the detection of PrP^{Sc} led to implementation of a variety of 'rapid', mostly enzyme-linked immunosorbent assay (ELISA)-based, tests, conducted on sub-samples of medulla oblongata, and these have become the principal approach for active surveillance diagnosis. Such tests provide a preliminary screening from which positive or inconclusive results are subject to examinations by IHC or Western blot confirmatory methods. Rapid test strategies are currently the main approach by which cases are detected and their wider use as part of the confirmatory process has been agreed in principle (15).

The use of a particular method will depend on the purpose to which the diagnosis is to be applied in the epidemiological context and its validation for that purpose. This range of purposes will extend from confirmation of the clinical diagnosis in the control of epizootic disease 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 of a clinical case or for screening of a population. 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 assessment (QA) are essential parts of the testing procedures and advice can be supplied by the OIE Reference Laboratories (15, 42). Whether BSE-infected animals are to be identified by passive or active surveillance, it is a 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 representative areas of the whole brain are examined. Moreover, 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 may prevent appropriate characterisation of the case, to confirm whether or not it is typical of BSE. 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.

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 selected for fixation for at least 5 days in 4% formaldehyde solution (i.e. 10% formal saline or 10% normal buffered formalin [NBF]) and subsequent histological processing by conventional paraffin wax embedding methods for neural tissue.

Fresh material for use in confirmatory immunoblotting to detect disease-specific PrP should be taken initially, as a complete coronal section (2–4 g) from the medulla, immediately rostral, or caudal, to the obex block taken for fixation. Alternatively the medulla, at the level of the obex, could be hemi-sectioned, as described for active surveillance (see below). All other brain areas should be subdivided by a sagittal paramedian cut (0.5 cm off the median). The smaller portion is reserved for the PrP^{Sc} detection by immunochemical methods (e.g. SAF-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, the brain is 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 small pieces of brainstem may be reduced to 2–5 days. However, evaluation of the effects of this kind of processing 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 two weeks' fixation.

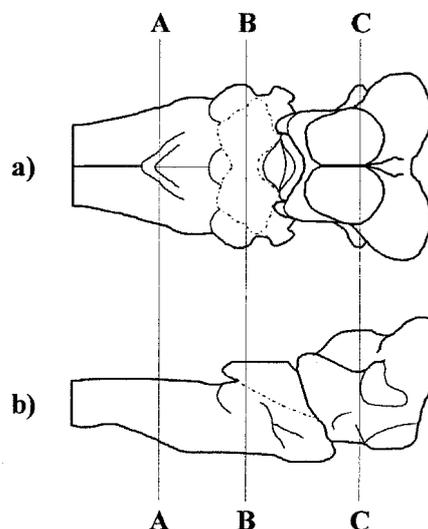


Fig 1. Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects.

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 phenotypic determinants, are consistent with that seen in the brains of cattle from the UK epizootic, it is adequate, although not ideal, for 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 (Fig. 2). Such instruments are available commercially, made of plastic or metal. It is possible that variations in technique, including orientation, are required with different forms of the instrument, thus highlighting the need for training of operators once there is agreement on equipment to be used. 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 fluid pressure (air or water) (20) 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 to be subjected to 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. Ideally, 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 much more comprehensive sampling of the brain of positive animals and enable the recommended approach to the characterisation of cases. This is particularly important if unvalidated tests 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, in the absence of a diagnosis of BSE having ever been made in a country, it is necessary to make provision for material to be available for further morphological and IHC examination that would allow identification of disease phenotype.

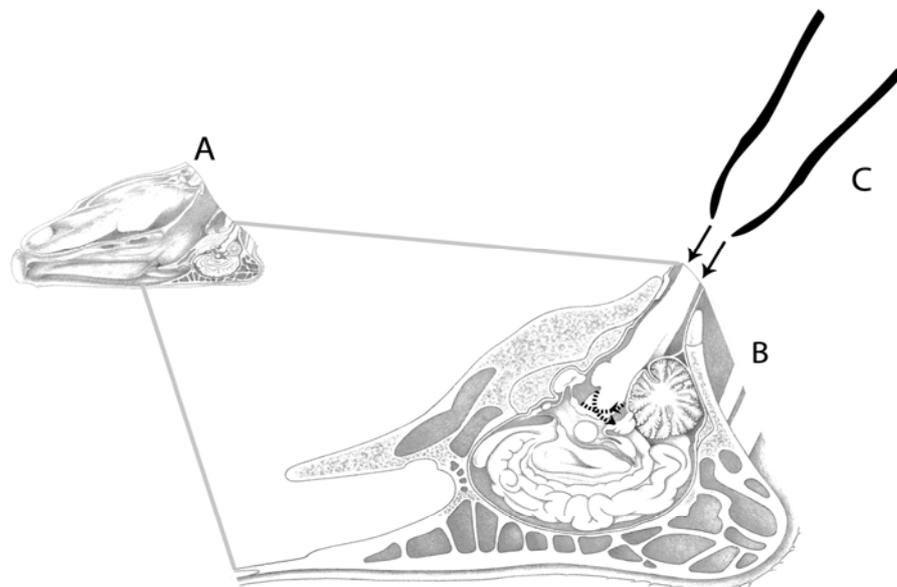


Fig. 2. After the head has been removed from the body by cutting between the atlas vertebra and the occipital condyles of the skull, it is placed on a support, ventral surface uppermost (A), with the caudal end of the brainstem (medulla oblongata) visible at the foramen magnum (see B, expanded drawing 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.

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

The sampling and processing of the brain tissue for use in the 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 fixed for immunohistochemistry/histology. 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 vacuolar changes, 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.

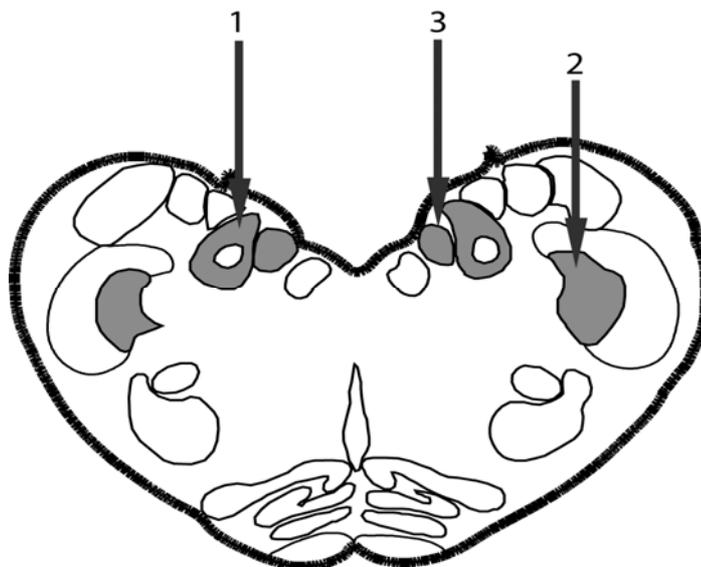


Fig 3. Cross section of the bovine brainstem at the level of the obex identifying the key target sites for diagnosis by histopathology and immunohistochemistry in BSE. These are principally the nucleus of the solitary tract [1] and the nucleus of the spinal tract of the trigeminal nerve [2]; but also 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 easily result in the complete loss of a target area for confirmatory testing, and significantly reduce the effectiveness of the 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. 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 some 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 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. 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 (30, 36) 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 high frequency of occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex, provides a satisfactory means of establishing a histopathological diagnosis on a single section of the medulla (34) in clinical suspect cases. 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 (18). The histopathological diagnosis of BSE must therefore not rely on the presence of vacuolated neurons alone, particularly in these anatomical locations.

The diagnosis may be confirmed if completely typical morphological changes are present in the medulla at the level of the obex, but, irrespective of the histopathological diagnosis, immunohistochemistry is now 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, this protocol, confined to examination of the medulla–obex, does not allow a full neuropathological examination for differential diagnoses to be established, nor does it allow a comprehensive phenotypic characterisation of any TSE. It is for this reason that it is recommended that whole brains are removed from all clinical suspects.

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

The universal use of PrP detection methods now provide a disease specific means of diagnosis independent of the morphological changes defined by the histopathological approach. Many laboratories have therefore now supplemented or replaced histopathological examination by IHC and 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 (12). Freezing prior to fixation will not compromise the immunoreactivity of a sample, but it may compromise the identification of target sites that need to be checked before a negative result can be recorded.

o **Immunohistochemical (IHC) methods**

The IHC examination to detect PrP^{Sc} accumulation is applied to sections cut from the same formalin-fixed paraffin-embedded material of medulla at the level of the obex as that used for the histopathological diagnosis (36). Several protocols have been applied successfully to the IHC detection of PrP^{Sc} for the diagnosis of BSE and although harmonisation toward a fully validated standardised routine diagnostic IHC method would seem desirable, experience has indicated that it is much 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 technique does not necessarily require lengthy tissue fixation, although for accuracy the guidelines established for histopathology still apply and, providing the tissue can be adequately processed histologically, it works well in autolysed tissues in which morphological evaluation is no longer possible (11, 25). However, it is still necessary to be able 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} (28). 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, it provides simultaneous evaluation or confirmation of this aspect of the disease phenotype. Current methods are available by reference to the OIE Reference Laboratories (15, 42).

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 pre-clinical diagnosis by biopsy techniques.

o **Western blot methods**

Immunoblotting techniques, are carried out on fresh (unfixed) tissue, and can be applied successfully even when tissue is autolysed (19). The SAF-immunoblot (15) 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 or dismissal of a BSE suspicion. In the last decade, alternative methods have been developed that are less time-consuming and less costly. Most of these techniques use a precipitation of PrP^{Sc} using phosphotungstic acid (PTA) or by other chemicals (31), and some are commercially available.

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.

o **Rapid tests methods**

Automated rapid Western blot 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 [14]).

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 (14, 26). The OIE now also has an approval process and protocols for such evaluations are posted on the OIE web site (43), 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 remain to be determined. Acknowledgement of this is particularly important in evaluating rapid test performance when testing animals that are not presenting clinical signs of BSE. 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. Rapid tests provide a means of initial screening for animals in the last few months of the incubation period, 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. In some countries, given the speed with which results can be obtained, the rapid tests are the preferred primary test, but confirmation of a diagnosis of BSE ideally requires either the examination of fixed brain by histopathology and/or immunohistochemistry or the application of an appropriate Western blot protocol. Nevertheless, 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, even if not ideal, for rapid tests to be used in combination for both primary screening in active or passive surveillance programmes and subsequent confirmation. It will be 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 VLA web site to assist those who wish to resort to this approach instead of histopathology and immunohistochemistry, or SAF immunoblot for confirmation (15). VLA will not change this web site without informing the OIE. The ideal combinations should include an ELISA and western blot method as they generate useful complementary data to assist in phenotypic characterisation of the sample in the absence of examination of fixed tissue.

Under certain circumstances, an EU or OIE approved rapid test could be used for the confirmation of BSE in bovines following an initial reactive result with an approved rapid test. Such approval is dependent on a review of reagents used in each rapid test to ensure that the pairs of tests used are compatible. On the basis of confidential data released by test manufacturers, a procedure is now available and is summarised below

1. The confirmation must always be carried out in a National Reference Laboratory (NRL) for TSEs.
2. The second test must include a negative control and a bovine BSE sample as positive control.
3. The second test must be a different test (in other words, two positive results involving the same test is insufficient for confirmation).
4. If a rapid Western blot is used as the first test, this result must be documented and submitted to the NRL.
5. One of the two methods must be a Western blot.

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-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 (32) 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 (9). 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 intracerebral/ intraperitoneal inoculation or by feeding of mice with brain tissue from terminally affected cattle, but bioassay is impractical for routine diagnosis because of the long incubation period. 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 test for BSE that can be applied to the live animal and has a sensitivity capable of detecting PrP^{Sc} at the low levels that may occur 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 (16). 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 (22, 29), has not been investigated for the diagnosis of BSE.

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 AND DIAGNOSTIC BIOLOGICALS

There are no biological products available currently. As discussed previously, diagnostic kits have been licensed for use in many countries.

REFERENCES

1. ANDERSON R.M., DONNELLY C.A., FERGUSON N.M., WOOLHOUSE M.E.J., WATT C.J., UDY H.J., MAWHINNEY S., DUNSTAN S.P., SOUTHWOOD T.R.E., WILESMITH J.W., RYAN J.B.M., HOINVILLE L.J., HILLERTON J.E., AUSTIN A.R. & WELLS G.A.H. (1996). Transmission dynamics and epidemiology of BSE in British cattle. *Nature*, **382**, 779–788.
2. BARON T.G.M., BIACABE A-G., BENCSIK A. & LANGEVELD J.P.M. (2006). Transmission of new bovine prion to mice. *Emerging Infect. Dis.*, **12**, 1125–1128.
3. BIACABE A.G., LAPLANCHE J.L., RYDER S. & BARON T. (2004). Distinct molecular phenotypes in bovine prion diseases. *EMBO Reports*, **5**, 110–114.
4. BRUCE M.E. (1996). Strain typing studies of scrapie and BSE. *In: Methods in Molecular Medicine: Prion Diseases*, Baker H. & Ridley R.M., eds. Humana Press, Totowa, New Jersey, USA, 223–236.
5. BUSCHMANN A., GRETZSCHEL A., BIACABE A.G., CORONA C., HOFFMANN C., EIDEN M., BARON T., CARAMELLI M., CONRATHS F.J. & GROSCHUP M.H. (2006). Atypical BSE cases in Germany. *Vet. Microbiol.*, **117**, 103–116.
6. BRUCE M.E., WILL R.G., IRONSIDE J.W., MCCONNELL I., DRUMMOND D., SUTTIE A., MCCARDLE L., CHREE A., HOPE J., BIRKETT C., COUSENS S., FRASER H. & BOSTOCK C.J. (1997). Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498–501.
7. CASALONE C., CARAMELLI M., CRESCIO M.I., SPENCER Y.I. & SIMMONS M.M. (2006). BSE immunohistochemical patterns in the brainstem: a comparison between UK and Italian cases. *Acta Neuropathol.*, **111**, 444–449.
8. CASALONE C., ZANUSSO G., ACUTIS P., FERRARI S., CAPUCCI L., TAGLIAVINI F., MONACO S. & CARAMELLI M. (2004). Identification of a second bovine amyloidotic spongiform encephalopathy: Molecular similarities with sporadic Cruetzfeldt-Jacob disease. *Proc. Natl Acad. Sci. U.S.A.*, **101**, 3065–3670.
9. CHAPLIN M.J., ALDRICH A.D & STACK M.J (1998). Scrapie associated fibril detection from formaldehyde fixed brain tissue in natural cases of ovine scrapie. *Res. Vet. Sci.*, **64**, 41–44.
10. COLLINGE J., SIDLE K.C.L., MEADS J., IRONSIDE J. & HILL A.F. (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature*, **383**, 685–690.
11. DEBEER S.O.S., BARON T.G.M. & BENCSIK A.A. (2001). Immunohistochemistry of PrP^{Sc} within bovine spongiform encephalopathy brain samples with graded autolysis. *J. Histochem. Cytochem.*, **49**, 1519–1524.
12. DEBEER S.O.S., BARON T.G.M. & BENCSIK A.A. (2002). Transmissible spongiform encephalopathy diagnosis using PrP immunohistochemistry on fixed but previously frozen brain samples. *J. Histochem. Cytochem.*, **50**, 611–616.
13. EUROPEAN COMMISSION (EC). Outcomes of discussions of the Scientific Steering Committee (1998–2003). http://ec.europa.eu/food/fs/sc/ssc/outcome_en.html

14. EUROPEAN COMMISSION (EC). TSE Community Reference Laboratory – Test Evaluation and approval. <http://www.defra.gov.uk/corporate/vla/science/science-tse-rl-tests.htm>
15. EUROPEAN COMMISSION (EC). TSE Community Reference Laboratory – Web Resources. <http://www.defra.gov.uk/corporate/vla/science/science-tse-rl-web.htm>
16. EUROPEAN FOOD SAFETY AUTHORITY (EFSA). Opinions of the Scientific Panel on Biological Hazards. http://www.efsa.europa.eu/en/science/biohaz/biohaz_opinions.html
17. GAVIER-WIDEN D., STACK M.J., BARON T., BALACHANDRAN A. & SIMMONS M. (2005). Diagnosis of transmissible spongiform encephalopathies in animals: a review. *J. Vet. Diagn. Invest.*, **17**, 509–527.
18. GAVIER-WIDEN D., WELLS G.A.H., SIMMONS M.M., WILESMITH J.W. & RYAN J.B.M. (2001). Histological observations on the brains of symptomless 7-year-old cattle. *J. Comp. Path.*, **124**, 52–59.
19. HAYASHI H., TAKATA M., IWAMARU Y., USHIKI Y., KIMURA K.M., TAGAWA Y., SHINAGAWA M. & YOKOYAMA T. (2004). Effect of tissue deterioration on postmortem BSE diagnosis by immunobiochemical detection of an abnormal isoform of prion protein. *J. Vet. Med. Sci.*, **66**, 515–520.
20. HEJAZI R. & DANYLUK A.J. (2005). Brainstem removal using compressed air for subsequent bovine spongiform encephalopathy testing. *Can. Vet. J.*, **46**, 436–437.
21. JACOB J.G., LANGEVELD J.P.M., BIACABE A.-G., ACUTIS P.-L., POLAK M.P., GAVIER-WIDEN D., BUSCHMANN A., CARAMELLI M., CASALONE C., MAZZA M., GROSCHUP M., ERKENS J.H.F., DAVIDSE A., VAN ZIJDERVELD F.G. & BARON T. (2007). Molecular discrimination of atypical Bovine Spongiform Encephalopathy strains in a geographical region spanning a wide area in Europe. *J. Clin. Microbiol.*, **45**, 1821–1829.
22. KARIV-INBAL Z., BEN-HUR T., GRIGORIADIS N.C., ENGELSTEIN R. & GABIZON R. (2006). Urine from scrapie-infected hamsters comprises low levels of prion infectivity. *Neurodegener. Dis.*, **3**, 123–128.
23. KIRKWOOD J.K. & CUNNINGHAM A.A. (2006). Portrait of Prion Diseases in Zoo Animals. *In: Prions in Humans and Animals*. Hörnlimann B., Riesner D. & Kretzschmar H. eds. De Gruyter, Berlin, Chapter 20, 250–256.
24. KONOLD T., BONE G., RYDER S., HAWKINS S.A., COURTIN F., BERTHELIN-BAKER C. (2004). Clinical findings in 78 suspected cases of bovine spongiform encephalopathy in Great Britain. *Vet. Rec.*, **155**, 659–666.
25. MONLEON E., MONZON M., HORTELLS P., VARGAS A., BADIOLA J.J. (2003). Detection of PrP^{Sc} in samples presenting a very advanced degree of autolysis (BSE liquid state) by immunocytochemistry. *J. Histochem. Cytochem.*, **51**, 15–18.
26. National Veterinary Assay Laboratory. Ministry of Agriculture, Forestry and Fisheries. Outline of Regulation System of Veterinary Drugs in Japan. <http://www.nval.go.jp/info/outline060728.pdf>
27. PRINCE M.J., BAILEY J.A., BARROWMAN P.R., BISHOP K.J., CAMPBELL G.R. & WOOD J.M. (2003). Bovine spongiform encephalopathy. *Rev. sci. tech. Off. int. Epiz.*, **22**, 37–60 (English); 61–82 (French); 83–102 (Spanish).
28. SCHALLER O., FATZER R., STACK M., CLARK J., COOLEY W., BIFFIGER K., EGLI S., DOHERR M., VANDELDELDE M., HEIM D., OESCH B. & MOSER M. (1999). Validation of a Western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). *Acta Neuropathol. (Berl.)*, **98**, 437–443.
29. SERBAN A., LEGNAME G., HANSEN K., KOVALEVA N. & PRUSINER S.B. (2004). Immunoglobulins in urine of hamsters with scrapie. *J. Biol. Chem.*, **279**, 48817–48820.
30. SIMMONS M.M., HARRIS P., JEFFREY M., MEEK S.C., BLAMIRE I.W.H. & WELLS G.A.H. (1996). BSE in Great Britain: consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases. *Vet. Rec.*, **138**, 175–177.
31. STACK M.J. (2004) Western immunoblotting techniques for the study of transmissible spongiform encephalopathies. *In: Methods and Tools in Biosciences and Medicine – Techniques in Prion Research*, Lehmann S. & Grassi J., eds. Birkhäuser Verlag, Berlin, Germany, 97–116.
32. STACK M.J., KEYES P. & SCOTT A.C. (1996). The diagnosis of bovine spongiform encephalopathy and scrapie by the detection of fibrils and the abnormal protein isoform. *In: Methods in Molecular Medicine: Prion Diseases*, Baker H. & Ridley R.M., eds. Humana Press, Totowa, New Jersey, USA, 85–103.

33. TAYLOR D.M. (2000). Inactivation of transmissible degenerative encephalopathy agents: A review. *Vet. J.*, **159**, 10–17.
34. WELLS G.A.H., HANCOCK R.D., COOLEY W.A., RICHARDS M.S., HIGGINS R.J. & DAVID G.P. (1989). Bovine spongiform encephalopathy: diagnostic significance of vacuolar changes in selected nuclei of the medulla oblongata. *Vet. Rec.*, **125**, 521–524.
35. WELLS G.A.H. & HAWKINS S.A.C. (2004). Animal models of transmissible spongiform encephalopathies: experimental infection, observation and tissue collection. *In: Techniques in Prion Research*. Lehmann S. & Grassi J., eds. Birkhäuser Verlag, Switzerland, 37–71.
36. WELLS G.A.H. & WILESMITH J.W. (1995). The neuropathology and epidemiology of bovine spongiform encephalopathy. *Brain Pathol.*, **5**, 91–103.
37. WELLS, G.A.H. & WILESMITH, J.W. (2004). Bovine spongiform encephalopathy and related diseases. *In: Prion Biology and Diseases, Second Edition*. Prusiner S., ed. Cold Spring Harbor Laboratory Press, New York, USA, 595–628.
38. WILESMITH J.W., HOINVILLE L.J., RYAN J.B.M. & SAYERS A.R. (1992). Bovine spongiform encephalopathy: aspects of the clinical picture and analyses of possible changes 1986–1990. *Vet. Rec.*, **130**, 197–201.
39. WILESMITH J.W., WELLS G.A.H., CRANWELL M.P. & RYAN J.B.M. (1988). Bovine spongiform encephalopathy: epidemiological studies. *Vet. Rec.*, **123**, 638–644.
40. WILL R.G., IRONSIDE J.W., ZEIDLER M., COUSENS S.N., ESTIBEIRO K., ALPEROVITCH A., POSER S., POCCHIARI M., HOFMAN A. & SMITH P.G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*, **347**, 921–925.
41. WORLD ORGANIZATION FOR ANIMAL HEALTH (OIE). World animal health situation – Bovine spongiform encephalopathy. http://www.oie.int/eng/info/en_esb.htm
42. WORLD ORGANIZATION FOR ANIMAL HEALTH (OIE). OIE Expertise – Reference Laboratories. http://www.oie.int/eng/OIE/organisation/en_LR.htm
43. WORLD ORGANIZATION FOR ANIMAL HEALTH (OIE). OIE: Validation and certification of Diagnostic Assays. http://www.oie.int/vcda/eng/en_background_vcda.htm
44. YAMAKAWA Y., HAGIWARA K., NOHTOMI K., NAKAMURA Y., NISHIJIMA M., HIGUCHI Y., SATO Y., SATA T. & EXPERT COMMITTEE FOR BSE DIAGNOSIS (2003). Atypical proteinase K-resistant prion protein (PrPres) observed in an apparently healthy 23-month-old Holstein steer. *Jpn. J. Infect. Dis.*, **56**, 221–222.

*

* *

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).