参考資料28

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Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

CHAPTER 2.3.13.

BOVINE SPONGIFORM ENCEPHALOPATHY

SUMMARY

The current 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 other countries are 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. A ban on the feeding of ruminant-derived protein to ruminants was first implemented in GB in July 1988. Since then, the feeding of mammalian-derived protein to ruminants has, with certain exemptions, been prohibited throughout the European Union and some other countries. From April 1996, this ban, with respect to mammalian meat-and-bone meal, was extended in the United Kingdom (UK) to all farmed food animals. Commission Decision 2000/766/EC of December 2000, included a temporary ban on the feeding of processed animal proteins to farmed animals kept for the production of food. Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposure to brain tissue from affected cattle. As a result of control measures, the epizootics in some European countries, including the UK and Switzerland, are already in decline. Cases of BSE currently occur throughout most of Europe and have now also been detected in Asia and North America.

The BSE agent is also believed also to be the common source of transmissible spongiform encephalopathies (TSEs) in several other species of bovidae and in species of felidae. There is evidence of a causal link between the BSE agent and a new variant form of the human

TSE, Creutzfeldt-Jakob disease (CJD).

BSE, as it occurs in GB, 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 sufficiently distinctive to lead to suspicion of disease, particularly if differential diagnoses are eliminated. Early clinical signs may be subtle, 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 slaughtered, the brain examined and the carcass destroyed. Confirmation of the diagnosis is based primarily on immunohistochemical (IHC) examination of the brain. Lesions have been described only in the central nervous system (CNS). 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: *No diagnostic test for the BSE agent in the live animal is presently available. The nature of the agents causing the TSE is unresolved. A disease-specific partially protease-resistant isoform of a membrane protein PrP ($PrP^{res;}$ PrP: Prion protein) has a critical importance in the pathogenesis of these diseases and according to the prion hypothesis is the sole component of the infectious agent.*

To confirm the diagnosis of spongiform encephalopathy, histological and immunohistochemical examination of the brain is necessary. The correlation between the clinical diagnosis and the neurohistological diagnosis in BSE can, with appropriate experience of both clinical signs and pathological changes, be greater than 90%. The histopathological examination may also provide a differential diagnosis in clinically suspect cases in which lesions of BSE are not detected. The pathognomonic lesion is a combination of both spongiform change in grey matter neuropil and neuronal vacuolation of certain brainstem nuclei. This change is usually, but not invariably, bilaterally symmetrical. Detection of accumulations of abnormal PrP (PrP^{res}) in the CNS of affected cattle by immunochemical methods offers a disease-specific diagnostic approach. PrP^{res} can be detected in unfixed brain extracts by immunoblotting and other enzyme immunoassay methods. Characteristic patterns of accumulations of disease-specific PrP in formalin-fixed affected brain can be demonstrated by immunohistochemical methods. Both approaches are now widely used as confirmatory diagnostic methods and are recommended as adjuncts to histological examination. A negative diagnosis should not be made solely on the basis of the absence of detectable vacuolation. IHC and other PrP detection methods can provide a BSE diagnosis in animals that appear clinically normal with minimal (or no) spongiform lesions in the brain. Characteristic fibrils, homologous with scrapie-associated fibrils and composed of PrP^{res}, can be visualised by electron microscopic examination of detergent-treated extracts of unfixed (or formalin-fixed) BSE-affected brains and have also been used to confirm the diagnosis, but lack the diagnostic sensitivity of the standard immunochemical methods.

BSE can be transmitted from brain tissue of terminally affected cattle to mice by intracerebral/ intraperitoneal inoculation or by feeding, but incubation periods of several months precludes bioassay from routine use. This is the only practical method currently available for detection of infectivity.

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 used for diagnosis of BSE in many countries.*

A. INTRODUCTION

A detailed account of bovine spongiform encephalopathy (BSE), its experimental transmissibility (20, 39), occurrence, epidemiology, clinical signs, pathology, diagnosis, prevention, and control has been given previously in English, French and Spanish (5655). More recent reviews have provided updated information (2, 16, 17, 26, 28, 36, 46, 62, 65, 80, 81, 96). BSE is an invariably fatal disease of domestic cattle, cases of which were first recognised in Great Britain (GB) in November 1986 (92). BSE has been shown to belong to that group of disorders known as the transmissible spongiform encephalopathies (TSEs) or prion diseases, typified in animal species by scrapie of sheep. These diseases are defined by the pathological accumulation, principally in the central nervous system (CNS) but also in lymphoreticular and peripheral nervous tissues, of an abnormal partially protease-resistant isoform of a host-encoded protein (PrP^{C}), designated PrP^{res}. Retrospective studies have indicated that the first cases of BSE presented around April 1985. The initial epidemiological studies established that the occurrence of BSE 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.

BSE has occurred in several countries other than the United Kingdom (UK) involving imported and/or indigenous cattle. The origin of such cases is 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 (24, 25, 31). Indeed, in some countries, the only cases detected reflect indigenous exposure rather than direct linkage with imported contaminated feed. Cases of BSE within indigenous cattle populations outside the UK have now been recorded in most European and some other countries where efficient surveillance or monitoring systems have been applied. These include Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Ireland, Israel, Italy, Japan, Liechtenstein, Luxembourg, the Netherlands, Poland, Portugal, Slovakia, Slovenia, Spain and,Switzerland. For current statistics on BSE around the world, readers should refer consultto the

OIE Web site.

From July 1988 in GB and from January 1989 in Northern Ireland, the feeding of ruminant-derived protein to ruminants was prohibited. With some exceptions, a ban on the feeding of mammalian-derived protein to ruminant animals has since been introduced elsewhere: in Switzerland (December 1990) (86) and throughout the European Union (June 1994) (62). From April 1996, this European Union ban, at least when protein was presented in the form of meat-and-bone meal, was extended in the UK to all farmed food animals, including horses and fish. From 1 January 2001 the use of mammalian meat-and-bone meal and fishmeal was prohibited throughout the European Union (32).

Experimentally it has been demonstrated that BSE can be transmitted to cattle following parenteral and oral exposure to brain tissue from affected cattle (20, 90). Epidemiological studies in Britain have revealed an increased risk for the offspring of clinical cases of BSE developing the disease themselves (27, 29, 30, 38, 98). Whether or not this is due to true maternal transmission has not been established. It is considered that this enhanced risk will not maintain endemic infection in the national (UK) cattle population, and indeed the estimates of increased risk for offspring of clinical cases have subsequently been revised downwards (26). There is no evidence of horizontal transmission of BSE between cattle. Epidemiological and transmission studies have not revealed evidence of a risk from semen (100) or milk (58, 84) or through embryos (100, 101). As a result of control measures, the epizootics in the UK and Switzerland (48) are in decline, others are showing the early effects of controls in the form of changes in age-specific incidence (24, 31, 65). In some countries the controls have not been in place long enough for the effects to be recognised. Interpretation of the status of epizootdemics in Europe has been enhanced by the introduction of active surveillance using rapid diagnostic tests, which have confirmed infection in cattle with clinical signs and also detected infected animals that have not been recognised as having reached clinical onset. Retrospective investigation at farms of origin frequently confirms that some signs have been presented before slaughter, but had not triggered consideration of a 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 suspected, or, in specific instances, known to have been caused by the BSE agent, or an agent indistinguishable from BSE by current available methods. Exposure is presumed to have been via feed.

In the past, epidemiological studies have found no connection between the exposure of humans to agents causing animal spongiform encephalopathies and the occurrence of the human TSE – Creutzfeldt-Jakob disease (CJD). In particular no enhanced occupational or dietary risk from exposure to sheep products has been established, thus suggesting that scrapie agents are not a human health hazard under natural conditions of exposure. However, monitoring of cases of CJD in the UK resulted, in March 1996, in the announcement of the recognition of ten cases of an apparently new variant of sporadic CJD

(v-CJD) in the UK (99). Strain-typing studies of the causal agent of v-CJD in mice (11, 76) have provided strong evidence that the same strain of agent derived from BSE is also found in v-CJD. Studies showing similarities between the Western- blot banding and glycosylation patterns of the disease-related isoform of PrP^{res} from patients with v-CJD and from certain animal species with naturally acquired or experimentally transmitted BSE, also support this conclusion (18, 49). Therefore, on present evidence, the most likely explanation of cases of v-CJD is exposure to the BSE agent, although the involvement of an agent indistinguishable from BSE by current methods cannot yet be excluded. The current incidence of the disease must still be viewed with caution. It is therefore now recommended that safety precautions for handling the BSE agent be based on the assumption that BSE is transmissible to humans. Because the incubation period of v-CJD is unknown, it is too early to predict the course of the epidemic; attempts to do so have produced widely varying predictions, although as cases continue to appear, the confidence limits are narrowing (19, 37, 41–43, 51, 59, 85).

Clinical BSE can be seen in adult cattle, and most cases occur in animals 4–5 years of age. There is no breed predilection, but the incidence of affected herds by functional type is much greater for dairy than beef, as, in the UK, it was mainly calves from dairy herds that were fed on concentrate rations containing meat-and-bone meal. 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 (9, 55, 92, 97). Occasionally, a case will present with acute signs and then deteriorate rapidly (97), although frequency of observation is a significant factor in determining whether early clinical signs are missed. 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, pelvic 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 and behaviour, abnormalities of posture and movement, and aberrant sensation, but the most commonly reported nervous signs are 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 (87). 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 pectoral limbs and, with advancing severity of locomotor signs, generalised weakness, resulting in falling and recumbency, can dominate the clinical picture. Reports of reduced rumination (3, 5), also bradycardia and altered heart rhythm (4), 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 (95). Clinical signs are essentially similar in other countries where BSE has occurred (9). 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 the complete destruction of the carcasses of affected cattle (63). Early in the disease course, the signs may be subtle, variable and non-specific, 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.

Because of the link established between BSE and v-CJD, BSE and related TSE agents are now categorised, in respect to biohazard, with the human TSE (1). Consequently, veterinarians and laboratory workers conducting necropsies on BSE-suspect animals or handling tissues derived from such animals, must conduct the work under containment level 3, (see Chapter I.1.6), sometimes with derogations. There is no evidence that the pathogen is airborne. Therefore, HEPA filtration of laboratory air is not required. Additionally, TSE agents are not inactivated by conventional fumigants, so there is no need for the facility to be sealable to enable fumigation. These derogations are contained in codes of practice issued by the UK Advisory Committee on Dangerous Pathogens (ACDP) under the auspices of the UK Health and Safety Executive in support of British and European legislation to protect individuals at their place of work. Local risk assessment should always be carried out to allow for the nature of the work to be taken into consideration. It is important that appropriate protective clothing be worn and that a strict code of practice be followed to prevent exposure to the agent. Laboratories conducting work on BSE must comply with national biocontainment and biosafety regulations. 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/in^2. 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 (82). 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 (82, 83).

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The nature of the agents causing animal or human TSEs remains unresolved (71). The disease-specific modified form (PrP^{res}) of a host-coded, highly conserved, membrane protein (PrP^{C}) of unknown function, is the only disease-specific macromolecule identified in the scrapie-like diseases. PrP^{res} is also frequently referred to as PrP^{Sc} and PrPD in scrapie and PrP^{bse} in BSE. A substantial 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. The opposing view is that the agent is a virus or is virus-like and contains nucleic acid. The identification of multiple 'strains' or isolates of scrapie agents, with characteristic incubation periods and patterns of neuropathological change when transmitted to mice, is considered to be more in keeping with this latter hypothesis. Previous studies to determine the resistance of the agent to degeneration have been used to suggest that the TSE agents do not contain nucleic acids. However, critical analyses of the denaturation characteristics of TSE agents, including effects of ultraviolet and ionising irradiation, extremes of temperatures, autoclaving and a large range of chemical disinfectants, suggest that the data obtained are compatible with that obtained for small viruses (15). The molecular basis for strain variation is still unclear, although proponents of the prion hypothesis argue that it is not incompatible with the existence of distinct strains (66).

Characterisation of isolates by transmission of BSE to mice has shown that BSE is caused by a single major strain of agent that differs from characterised strains of the scrapie agent in sheep (10). Uniformity of the pathology among affected cattle has also supported the notion of a single BSE strain and enabled the definition of a particular disease phenotype for BSE (91). This specific pattern of neuropathology in the host species is an important feature of the case definition of BSE. It is difficult however to totally exclude the possibility that other strains of the agent may exist at low frequency. In the absence of *in-vitro* methods for isolation of the causative agent, the conventional basis of confirmation of the diagnosis in this group of diseases has been 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. However, given the essential role of the PrP^{res} molecule and increases in technical capabilities in this area, it is now important that diagnostic approaches use one or more methods for the detection of the abnormal form of the protein. The demonstration by electron microscopy on CNS extracts of characteristic fibrils, termed scrapie-associated fibrils (SAF), which are composed largely of PrP^{res}, is a further morphological diagnostic method. Methods of

disease-specific PrP detection include immunohistochemical (IHC) demonstration, Western blotting/SAF-immunoblotting and, most recently, a number of rapid immunoassays for screening. 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 pathological case definition adopted will also differ according to whether the method is to be applied for confirmation of a case or for screening of a population. For the former it is important to use approaches that can monitor the pathological phenotype of BSE. It is also clear that the performance of individual methods will be crucial to this process of selection of a single method or a portfolio approach. It must be stressed that the development of methods, particularly rapid screening immunoassays for the detection of PrP^{res}, is a rapidly evolving field. Care should be taken in the interpretation of data using methodologies that do not enable careful cross-referencing with the gold standards defined here. This is particularly important with respect to the definition of strain. Differences in methodology can, without appropriate comparison with previously published criteria, generate differences in results that do not justify notification of the identification of a new strain. Precipitate announcements of the detection of new strains can have serious repercussions, while on the other hand substantiated identification is important for policy and control purposes.

Quality control (QC) and quality assessment (QA) should form an essential part of the testing procedures. The OIE Reference Laboratories can be contacted to provide assistance in this area and to help develop inter-laboratory comparisons at the international level.

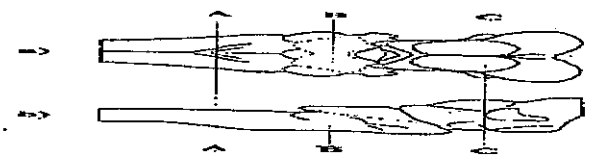
a) Sample preparation

For the preparation of material for diagnostic examination, cattle suspected of having the disease should be killed with an intravenous injection of a concentrated barbiturate solution following sedation, if necessary. The technical procedures concerned with collection, fixation, and histological processing have been described (8, 73) and are revised and summarised below.

In all circumstances of 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 important that a standard neuropathological approach be followed in which representative areas of the whole brain are examined. Departure from this is dependent on local national circumstances, including whether or not a differential

diagnosis is required. Additionally, where rapid immunoassays are selected as the primary method of choice, care will be needed in ensuring that sampling and tissue preparation for one test do not compromise the ability to confirm pathological phenotype by histopathological means.

Brain tissue should be removed as soon as possible after death. Fresh material for potential use in tests to detect disease-specific PrP should be taken ideally as a complete coronal section (2–4 g) from the medulla, caudal to the obex, specifically avoiding damage to the obex region. The cervical spinal cord and the lateral hemisphere of cerebellum also offer optimal sampling areas that will not encroach on histopathological requirements. This tissue is stored frozen prior to testing; precautions must be taken to insure that the tissues for histological or IHC examination are not frozen as this will provide artefactual lesions that may compromise the identification of vacuolation, and/or target site location. It is possible (but not desirable) to undertake immunohistochemistry for PrP on material that has been frozen prior to fixation (21). However, it is still important to be sure that target sites have been identified and checked before a negative results can be recorded. If the remaining whole brain is sampled for the histopathological examination, it should be placed in approximately 4–6 litres of 10% formol saline 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 into smaller coronal pieces, leaving intact the diagnostically important areas at the obex, the cerebellar peduncles and the rostral colliculi. Depending on some other factors (temperature, agitation, use of microwave) the fixation time for these small pieces of brainstem may be reduced to 2–5 days. The other formol-fixed parts of the brain may be used for differential diagnosis after completing the standard 2 weeks' fixation. Initially, a single block cut at the obex of the medulla oblongata (Fig. 1) should be selected for histological processing by conventional paraffin wax embedding methods for neural tissue. Sections, cut at 5 µm thickness and stained with haematoxylin and eosin, are examined for characteristic spongiform change and neuronal vacuolation. If results are inconclusive because of minimal lesions, or the material is histologically uninterpretable due to autolysis or damage, or if there are no histological lesions present, it is necessary to carry out additional tests, including immunohistochemistry (IHC) or immunoblotting.



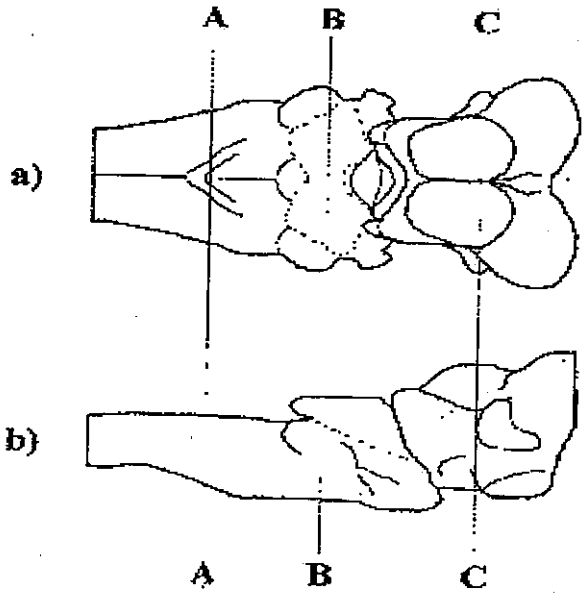


Fig. 1. *Brainstem after the removal of the cerebellum, from a) dorsal, and b) lateral aspects.*
Recommended levels at which sections should be taken:
A–A = medulla, at the obex;
B(B = medulla through caudal cerebellar peduncles;
C(C = midbrain through rostral colliculi.

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 is consistent with that seen in the brains of cattle from the UK epizootic, it is adequate for monitoring purposes to remove the hind brain alone (Fig. 1). This can be achieved via the *foramen magnum* without removal of the calvarium. This will reduce the amount of fixative required, thereby lowering costs and improving safety, while maintaining representation of the major target areas for histological examination. The diagnosis may be confirmed if completely typical changes are present in the medulla at the level of the obex. When lesions are not obvious in the medulla (obex), immunohistochemistry should be performed. However, given the constant lesion pattern, this is unlikely to contribute additional confirmation in more than 0.5% of cases of BSE where lesions are absent in the medulla (obex) section (88). Clearly this abridged protocol does not allow a full neuropathological examination for differential diagnoses to be established, nor does it represent a comprehensive phenotypic characterisation of any TSE.

Where the index case is identified through active surveillance, the necessary brain areas for full phenotypic characterisation may not be available. In most countries, hind brain alone is collected (see below), even before the first confirmation of BSE.

Ideally, provision should be made for heads which 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 this recommended approach to the characterisation of cases. This is particularly important if un-validated tests are used, and where in the absence of direct comparison with the methods described here results in claims that new strains have been identified.

The 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 for the variant methodology. The preferred sample for immunoassay should be at, or within 1.5 cm anterior to, the obex. The choice of target site should take into account the preferred method of confirmation, where the inability to examine brainstem histologically at the obex may prevent the detection of bilateral vacuolation. Sampling the rostral medulla for rapid test does not compromise examination by histological or immunohistochemical means. Hemisection of the brainstem at the level of the obex will result in loss of the ability to assess the symmetry of lesions, but the need for such assessment is less if immunohistochemistry is used. If this approach is adopted however, it becomes critical to ensure that the target site is not compromised. Both the dorsal nucleus of the vagus nerve (the target area for scrapie) and the nucleus of the solitary tract (the target area in cattle) are small, and lie close to midline (Fig. 2).

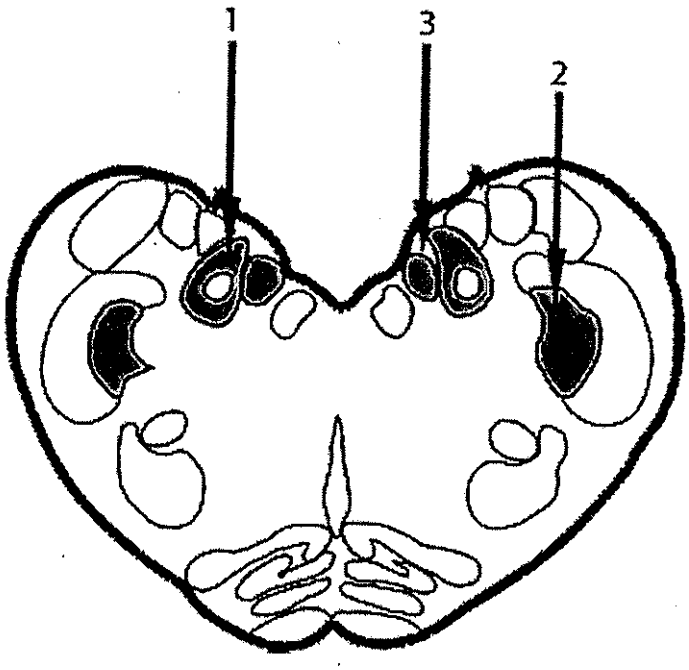


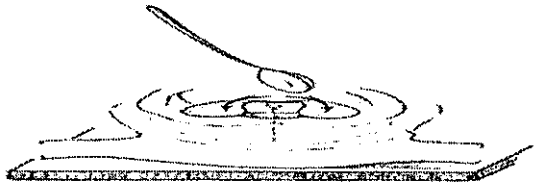
Figure 2. *Cross section of the brain-stem at the level of the obex*

identifying the key target sites
for diagnosis by histopathology and immunohistochemistry in BSE
(nucleus of the solitary tract [1] *and the nucleus of the trigeminal tract V* [2]) *and scrapie*
(dorsal nucleus of the vagus). [3]).

Inaccurate hemisecting could easily result in the complete loss of target area for confirmatory testing, and significantly reduce the effectiveness of the surveillance programme. Such an approach needs to be implemented with a very clear policy and monitoring programme for training and QA of sampling procedures. Because of the uneven distribution of PrP^{res}, sample size should be as specified in the diagnostic kit or if not specified should be at least 0.5 g. Performance characteristics of all of the tests may be compromised by autolytic changes. In order to reduce hazard to the operators collecting large numbers of samples for an active surveillance programme, bovine brains should be sampled without opening the cranium. This is readily achieved, even at abattoirs, following training of operators in the use of a specially designed spoon, which can be inserted through the foramen magnum of the severed head. The following is a protocol that has been drafted by the OIE Reference Laboratory, Bern, Switzerland. Some rapid test manufacturers also sell disposable spoons for brain removal.

- **Removal of the brainstem**

After the head has been separated from the body between the atlas and foramen magnum, the head is put on a support with the frontal bone down; the caudal end of the brainstem is visible through the foramen magnum. The brainstem is dissected through the foramen magnum without opening the skull by means of a a specially designed spoon with sharp edges and a long handle (Fig. 3). The spoon is inserted into the foramen magnum between the brainstem and the bone and moved along the wall of the skull moving to the left and the right to severe the cranial nerves on both sides, while avoiding damage to the brain tissue by keeping close to the bone. The spoon is advanced for a distance of approximately 7 cm in this fashion and then bent downwards cutting and separating the caudal medulla oblongata (with some fragments of cerebellum) from the rest of the brain. The spoon – remaining in a bent downward position – is then pulled towards the operator. In this way the severed brainstem slips out of the skull through the foramen magnum.



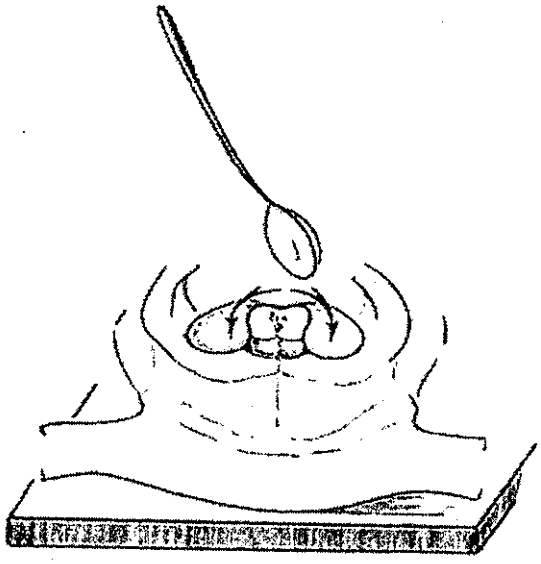


Fig. 3. *The head is separated from the body and placed on a support upside down; the brainstem (bs) is separated from the bone with cutting movements left and right (curved arrows) by means of a long-handled specially designed spoon with sharp edges, inserted in the foramen magnum between bone and brain tissue. The preferred sample for immunoassay should be at, or within 1.5 cm anterior to the obex.*

The initial pathological case definition of BSE was based on the histopathological changes in the CNS, and this has provided the usual basis for confirmation of the clinical diagnosis of BSE (88, 92). The histopathological examination also allows confirmation of the characteristic neuropathological phenotype of BSE (78, 94). The histopathological changes are neurodegenerative and closely resemble those of scrapie in sheep. The most prominent features are vacuolar and comprise a spongiform change in the neuropil of grey matter in specific neuroanaotomical areas, and single or multiple vacuoles within neuronal perikarya. The precise appearance of the spongiform change in TSEs, as observed by light microscopy, has been defined previously (56). In BSE, spongiform change is the predominant form of vacuolar change. Both forms of vacuolation are bilaterally distributed and usually symmetrical with a consistent pattern of severity relative to distribution throughout the brain (89, 94). The high frequency of occurrence of neuroparenchymal vacuolation in certain anatomic nuclei of the medulla oblongata at the level of the obex has, in the BSE epizootic in the UK, provided a satisfactory means of establishing a diagnosis on a single section of the medulla (88). However, observation of equivocal lesions in the medulla at this level requires examination of other brain areas to detect cases of BSE with minimal or potentially atypical lesions and, when necessary, to establish pathological differential diagnoses.

Neurodegenerative changes other than vacuolation are not prominent in BSE. A gliosis (astrocytosis), as seen in scrapie, is another feature, particularly in sites of vacuolar change. Detection of gliosis is assisted by the use of special stains and immunohistochemistry. For example, astrocytosis can be demonstrated by the immunohistochemical detection of increased glial fibrillary acidic protein (GFAP).

The interpretation of observed vacuolar changes in the bovine brain must be approached with caution. Vacuoles within the perikarya, indistinguishable from those of BSE, have been reported in neurones of the red and oculomotor nuclei of the midbrain and other brainstem nuclei as an incidental finding in cattle (34, 40, 57, 92). Thus, like the diagnosis of scrapie, which may be confounded by the occurrence of such neuronal vacuolation scattered in the medullae of healthy sheep (see Chapter 2.4.8. Scrapie) (86, 87), histopathological diagnosis of BSE must not rely on the presence of occasional solitary vacuolated neurons. Even relatively numerous vacuolated neurons in the red nucleus and in the habenular nuclei must be disregarded. The presence of spongiform change in the neuropil in specific neuroanatomical locations in BSE provides the most confidence of minimising false-positive diagnoses.

As with scrapie of sheep, the possibility of BSE cases occurring in which brain lesions are minimal or undetectable by light microscopy, is a potential problem that can be resolved only by diagnostic criteria independent of histopathology (73, 93). (See also the next section.)

c) Detection of disease-specific forms of PrP

In the past in many countries histopathological examination confined to the medulla oblongata is the laboratory investigational method that has been used to handle large numbers of suspect cases. Demonstration of typical changes provides a definitive diagnosis. Many laboratories have now supplemented or replaced histopathological examination by IHC and other PrP PrP-detection methods. When the results of the histopathological examination are inconclusive or negative, or the brain material is unsuitable for histological examination because of autolysis or damage, detection of abnormal accumulation of PrP becomes the method of choice. Applying these PrP tests becomes increasingly important in the decaying phase of the epizootic and in surveillance programmes where critical monitoring of disease is required.

In conjunction with, or even as an alternative to, the histopathological evaluation of medulla sections is the use of IHC to detect PrP^{res} accumulation in formalin-fixed, paraffin-embedded material (47, 94). Several protocols have

been applied successfully to the IHC detection of PrP for the diagnosis of BSE (44, 47, 54, 94). Harmonisation toward a fully validated standardised routine diagnostic IHC method is desirable. However, it is likely that only the general principles can be prescribed, with precise methods being determined by each individual laboratory. A European Commission (EC) funded collaboration among European laboratories, which addressed the need for harmonisation of diagnostic methods, concluded that the total standardisation of methods was difficult and possibly unnecessary. Local conditions will always dictate a degree of inter-laboratory variation, and each method should be optimised for use with the standard tissues and common reagents (such as water) used locally. Historically there has also been a dependence on 'in-house' polyclonal antibodies, but the increase in commercially available monoclonal antibodies has reduced this variation significantly. It is much more important to achieve a standardised output, as monitored by participation in QA exercises, and by comparison with the results of a standardised model method.

The technique is more sensitive than routine histopathology as it can detect cases in the last months of incubation before the occurrence of vacuolar changes, at least in experimentally induced cases of BSE (62) and possibly also in the natural disease (23). BSE can therefore be diagnosed by IHC in animals with equivocal (or no) morphological changes. 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. IHC detection of abnormal PrP accumulations is as sensitive as the Western blotting method for detection of PrP^{res} (69). In combination with good histological preparations, IHC allows detection of abnormal PrP accumulations and, as this abnormal PrP accumulation, like the vacuolar pathology, exhibits a typical distribution pattern and appearance, it provides simultaneous evaluation or confirmation of the disease phenotype.

Those laboratories without previous immunohistochemical experience may wish to adopt the model method shown below. As with other techniques described in this text, it will be insufficient in itself to enable immediate replication in a laboratory. Nevertheless, in consultation with the authors and following specific modifications that are required to account for variations in reagent qualities or concentrations, it can be used for comparison with locally preferred methods. The method described below is that used by the OIE Reference Laboratory in the United Kingdom for the diagnosis of BSE, and for the preparation of reference materials for other laboratories.

- **Disease Disease-specific PrP – Iimmunohistochemical Immunocytochemical labelling**

  - **Reagents**

Store commercial preparations according to the manufacturer's instructions.

Tris buffered saline Tween 20 (0.5%), pH 7.6 (TBST)

Formic acid (96%)

Citrate buffer solution (0.2%), pH 6.4

Hydrogen peroxide (30% w/v) (Sigma)

Methanol

Vector Labs, Vectastain Elite RAT IgG avidin–biotin complex (ABC) kit

Copper sulphate solution (0.5%)

Di-amino-benzidine (DAB) (Sigma)

Mayer's haematoxylin

0.5% conc. HCl in absolute ethanol

Absolute ethanol

Xylene

Ammonia water

Primary antibody (rat anti-bovine PrP monoclonal R145 antibody, available from Veterinary Laboratoryies Agency, Weybridge, UK)

  - **Tissues**

Formalin Formalin-fixed tissues of maximum thickness of 3 mm. Immerse in 96% formic acid for 1 hour. Rinse in running water for 10 minutes. Immerse in neutral buffered formalin for 1 hour and routinely process to wax.

- **Method**

Following preparation of paraffin wax embedded blocks (according to routine methods), cut sections at 3 µm thickness and mount on polysine polysine-coated slides or equivalent. Air dry and place in 60°C oven overnight.

i) Deparaffinise sections in xylene for 10 minutes.

ii) Wash in absolute ethanol twice for 30 seconds (agitate).

iii) Wash in running tap water for 10 minutes.

iv) Immerse in 96% formic acid for 5 minutes. (Formic acid treatment is used for preliminary antigen retrieval, which also maintains good tissue morphology during subsequent autoclaving. It also increases operator safety by reducing infectivity levels in the tissue being handled.)

v) Wash in running tap water for 10 minutes.

vi) Immerse in citrate buffer solution in an open plastic trough and autoclave for 5 minutes at 121°C.

vii) Cool at room temperature for 10 minutes then rinse in running tap water for 10 minutes.

viii) Immerse in 3% hydrogen peroxide in methanol for 20 minutes.

ix) Rinse in running water for 10 minutes.

x) Dry sections and isolate with wax pen.

xi) Sections should not be allowed to dry from now on.

xii) Rinse sections in TBST twice.

xiii) Apply normal rabbit serum (NRS)* for 60 minutes at room temperature. (*Dilute NRS according to manufacturer's instructions.)

xiv) Drain off NRS and apply primary antibody (rat anti-bovine PrP monoclonal antibody R145 at 1/3000) for 16–18 hours at room temperature.

xv) Rinse sections in TBST twice for 2–3 minutes each time.

xvi) Apply secondary antibody* (rabbit anti-rat IgG) for 60 minutes at room temperature. (*Dilute secondary antibody according to manufacturer's instructions.)

xvii) Prepare avidin–biotin complex (ABC) reagent according to manufacturer's instructions and leave at room temperature for 30 minutes be before use.

xviii) Rinse sections in TBST twice for 3 minutes each time.

xix) Apply ABC for 30 minutes at room temperature.

xx) Rinse sections in TBST twice for 3 minutes each time.

xxi) Apply DAB – for 10 minutes.

xxii) Rinse in tap water twice for 2 minutes each time.

xxiii) Immerse in 0.5% aqueous copper sulphate for 5 minutes.

xxiv) Rinse in tap water for 1 minute.

xxv) Immerse sections in Mayers haematoxylin for 5 minutes.

xxvi) Rinse in tap water until water runs clear.

xxvii) Differentiate in 1% acid alcohol (1% HCl in ethanol) for 2–3 seconds.

xxviii) Rinse sections in tap water for 1 minute.

xxix) Immerse in alkaline ammonia water solution to convert brown haematoxylin counterstaining to blue. The same effect can be obtained by washing in running water for 5 minutes or by immersion in 0.05% lithium carbonate.

xxx) Immerse sections in absolute ethanol twice for 30 seconds each time.

xxxi) Clear sections in xylene for 2–5 minutes.

xxxii) Apply cover-slips and allow to dry.

Abnormal accumulations of PrP as shown by IHC are considered to have potential for the preclinical diagnosis of scrapie in sheep (in some, but not all, genotypes) using tonsillar (72) or nictitating membrane (64) lymphoid tissue biopsies. However, BSE infectivity has not been detected by mouse bioassay or IHC in lymphoid tissues at any time during the incubation period or clinical disease course (90), other than in distal ileum containing Peyer's patches in experimentally infected cattle. Currently, this suggests that these tissues are unlikely to be of use diagnostically. One unpublished result of the detection of infectivity in the tonsil of an experimentally infected animal, 10 months after oral exposure, remains incomplete. The finding is in contrast to negative results in tonsils collected at earlier and later time points in the study, including clinically affected animals, and does not indicate a breakthrough in the scope for *in-vivo* testing of cattle.

Detection of PrP^{res} by SAF-purification followed by immunoblotting techniques (35, 50, 79), is carried out on fresh (unfixed) or frozen brain or spinal cord material. Improvements in purification methods for extracting PrP (7, 22) have contributed to increased sensitivity of this method. Where sufficient sample size remains, this methodology frequently provides a sensitive method of confirming diagnosis following initial suspicion of disease using more recent rapid rapid tests (see below). For that reason, and in the absence of a full and accessible published method elsewhere, the method for SAF-immunoblotting is reproduced below.

- **Protocol for the SAF-Immunoblot for TSE diagnosis**

The following is an example of a protocol for the purification and detection of the disease specific isoform of the prion protein (PrP^{res}) from unfixed brainstem material. Purification of the scrapie-associated fibrils (SAF) is first achieved by ultracentrifugation followed by a proteinase K treatment to digest residual cellular PrP^{C}. Fibrils are then spun through a sucrose cushion for further purification prior to immunoblot detection of PrP^{res}.

- **Sample preparation**

Material must be taken from the brainstem and there, if available, from the obex region (this is the region with the highest PrP^{res} content). Samples should be taken as follows:

Negative control: 4 g

Positive control: 4 g

Suspect sample with strong rapid test signal (e.g. optical density [OD] >2.5 in Bio-Rad Platelia): 1 g, this material will be completely treated with Proteinase K (PK)*.

Suspect sample with weak rapid test signal (e.g. OD <2.5 in Bio-Rad Platelia): 2 g +PK and 2 g –PK treatment

*NOTE: Depending on the rapid test result, the amount of suspect brain stem material that is taken for confirmatory purposes may be either 2 g or 1 g. However, if a negative result occurs in a sample where only 1 g has been examined, this sample must be repeated with at least 2 g +PK treatment.

i) Take appropriate amount of brainstem material (obex region).

ii) Cut into small pieces (removing dura mater) and add 5 ml of brain lysis buffer (BLB) (10 g N-Llauroyl-sarcosine, sodium salt ([SIGMA #L5125)] in 100 ml of 0.01 M sodium phosphate buffer, pH 7.4, plus protease inhibitors – 10 µl of 100 mM Pphenylmethylsulfonylfluoride [(PMSF]) and 10 µl of 100 mM N-ethyl-maleimide [(NEM])).

iii) Homogenise thoroughly in a glass homogeniser (douncer).

iv) Carefully transfer into a 50 ml plastic tube.

v) Add another 2–5 ml of BLB into the douncer, rinse well and also add this also into the plastic tube, bringing the end volume of the homogenate to 10 ml. Sonify Sonicate for 1 minute.

vi) Transfer sample into quickseal tube, balance weight by adding BLB, and close tube with tube topper.

vii) Centrifuge at 20,000 ***g*** (17,000 rpm) for 30 minutes at 10°C in a 70 Ti Beckmann ultracentrifuge rotor.

viii) Carefully remove the supernatant with a syringe and add into a clean centrifuge tube. Then fill with BLB to the neck of the tube. Seal tubes as above and centrifuge at 177,000 ***g*** (46,000 rpm) for 135 minutes at 10°C in a 70 Ti Beckmann ultracentrifuge rotor.

ix) Discard the supernatant and suspend the pellet in 3 ml distilled water with 50 µl 1 M Tris/HCl, pH 7.4 (0.0167 M) by gentle aspiration with a pipette. For less than 2 g brain material, only 1.5 ml distilled water with 25 µl 1 M Tris/HCl, pH 7.4 (0.0167 M) is used.

x) Incubate in a water bath at 37°C for 15 minutes stirring regularly.

xi) MFor the KI-HSB solution, make up 1.5 g Ssodium thiosulphate, 1.0 g N-lauroyl-sarcosine in 1 ml 1 M Tris/HCl and add 10 g of potassium iodide for a 10% solution or 15 g for 15% solution. Dilute to 100 ml with distilled water. Add 6 ml of the 15% KI-HSB solution to the sample and incubate for a further 30 minutes as in step x. For less than 2 g brain material, 3 ml 15% KI-HSB is added.

xii) Divide the solution into two aliquots of 4.5 ml (only for samples that are divided into plus and minus PK treatment).

xiii) To one aliquot add 1 mg/ml PK and incubate for 1 hour as in step ix. Amount of PK solution to be added – for: 2 g and less: + 45 µl PK.

xiv) To the other aliquot, add 4.5 ml of 10% KI-HSB and then carefully transfer into an ultracentrifuge tube. Carefully add 2 ml 20% sucrose with a long syringe to the bottom of the tube and fill tube to neck with 10% KI-HSB and centrifuge at 189,000 ***g*** (51,000 rpm) for 1 hour at 10°C.

xv) Carry out step xiii on the PK -treated sample.

xvi) Carefully tip off supernatants and drain tubes well, always paying attention to the pellet.

xvii) Resuspend samples in 40 µl 1 x sample buffer - (2 ml of 20% sodium dodecyl sulphate [SDS)], 1 ml Tris/HCl ([1 M, pH 7.4] 1 ml mercaptoethanol, 0.6g sucrose, 1–3 drops bromphenol blue, 15 ml distilled water).

xviii) Sonify Sonicate samples for 30 seconds.

xix) Centrifuge briefly to concentrate the sample in the bottom of the tube.

- **SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western blot**

  i) Assemble a large and a small glass plate and spacers for a minigel after cleaning glass plates with 70% ethanol and drying them off completely.

  ii) Prepare separation gel and pour or pipette the gel between the plates up to 2 cm from the top of the small glass plate. Carefully pipette a layer of isopropanol on the top of the gel and let set for approx. 60 minutes.

  iii) Carefully rinse the gel surface with deionised water. Prepare the stacking gel and pour or pipette it on top of the separation gel. Insert a comb within 30 seconds and let the gel set for 5 minutes.

  iv) Pull the comb and assemble the gel apparatus.

  v) Fill 1 x electrophorese gel chamber with electrophoresis buffer (25 mM Tris ([3.03 g/litre], 192 mM glycine [(14.4 g/litre], 20% methanol [200 ml/litre], 0.2% SDS [2 g/litre]). Gel must be well below buffer level. Carefully clean the wells from gel rests with a pipette.

  vi) Load 30 µl of each sample on 13% SDS mini-gel after incubating samples for 5 minutes at 95°C.

  vii) Run gel at 100 V until samples have moved well into the separation gel, then continue at 200 V (will take another 30–40 minutes). Stop the gel run when blue colour front has just run out at the bottom of the gel.

  viii) Set transfer: on the semidry chamber, assemble in the following order:

  - three layers of wWhatman paper, slightly larger than the membranes in size, soaked well in blotting buffer;

  - immobilon-P-Transfer-membrane (this membrane must have been equilibrated first in methanol for 5 minutes and then in blotting buffer beforehand);

  - gel;

- three layers of Whatman paper soaked well in blotting buffer.

Close semi-dry chamber and run the transfer at 15 V for 50 minutes.

ix) Take membrane out of the transfer cell and block it in 20 ml I-Block (5% non -fat dried milk in phosphate buffered saline [PBS] + 0.1% Tween 20) for 30 minutes at room temperature on a rocker.

x) Incubate for 1.5 hours at room temperature or over night at 4°C with the primary antibody in I-Block.

xi) Wash three times for 10 minutes each in PBS + 0.1 % Tween 20 (PBS/Tween) on a rocker.

xii) Incubate for 1 hour with the conjugate (GAM-AP) diluted 1/000 in PBS/Tween at room temperature on a rocker.

xiii) Wash three times for 10 minutes each in PBS/Tween at room temperature on a rocker.

xiv) Incubate in 1 x assay buffer for 2 minutes at room temperature on a rocker.

xv) Visualise bound antibodies by adding 1.5 ml CDP Star detection reagent (Tropix) on each blot, incubate for 5 minutes, then wrap membrane in plastic foil and detect signals in a camera or by exposing a film.

- **Interpretation of results**

As this protocol aims at the concentration of the insoluble PrP^{res} by ultracentrifugation, no signal would be expected to be seen from the digested negative control sample and the undigested aliquot, containing only soluble PrP^{C}, is likely to give only a low signal. Prion protein is glycosylated twice. As a consequence after PK digestion PrP^{res} is yielding bands at m.m. 30–27 kDa, 26–24 kDa and 21–19 kDa in the immunoblot. For BSE PrP^{res} the upper band is most prominent. This is also true for most scrapie PrP^{res} signals, but banding intensities can also be different.

Negative control:

- Not treated with PK: no or only weak PrP^C (33–35 kDa) signal.

- Treated with PK: no PrP specific signal.

Positive control:

- Not treated with PK: very strong signal, often too strong to differentiate single PrP bands, highest signal intensity at 33–35 kDa;

- Treated with PK: three PrP^{res} bands visible.

A shift in molecular weight should be visible between the undigested and the digested fraction of the positive control to verify the PK activity.

Diagnostic sample:

- The PK -treated diagnostic sample is diagnosed as positive if PrP^{res} signals are clearly visible. Diagnostic samples should preferentially be loaded on the same blot as the PK-treated positive control. They must always be loaded on the same gel as an undigested PrP^C control sample to be able to judge the shift in molecular weight.

- The PK-treated diagnostic sample is diagnosed as negative if there is no detectable PrP^{res}-specific signal

- Testing must be repeated if the results of the positive or negative control are untypical or if the result of the diagnostic sample is inconclusive, e.g. as signals are:

  - very faint (repeat SAF preparation with a higher amount of brain material)

  - banding pattern does not match the positive control (repeat procedure, use other diagnostic methods in addition).

- **Rapid tests for the detection of the disease-specific forms of PRP**

Automated Western blot and enzyme-linked immunosorbent assay (ELISA) techniques have been developed which that allow screening of large numbers of brain samples (60, 61, 69, 70) and are now commercially available. Such techniques can be performed rapidly and are potentially more sensitive than the histopathological evaluation and may compare with that of SAF-immunoblot. Evaluation in comparison with the sensitivity of immunohistochemistry remains to be determined. In a trial conducted on behalf of the EC (60, 61), it was demonstrated that one such Western immunoblot method, or either of two specific ELISA methods, when evaluated on brain tissue, were suitable for diagnostic use in specifically targeted populations. This evaluation was 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. These tests are now approved and used in European and some other countries for large-scale screening and surveillance programmes. They provide a means of initial screening for animals in the late stages (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 recently developed 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 requires examination of fixed brain by histopathology and/or IHC.

A further generation of rapid diagnostic tests was evaluated by the EC in 2001 (33, 70). The process has highlighted the need for such an evaluation process, and identified the dangers of using research tools prematurely for active surveillance. Although the evaluation programme is in support of European legislation on surveillance for BSE, the consequences are of relevance to other countries as well. 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. The studies initiated by the EC represent evaluations of the tests: test validation is currently ongoing. Caution must be exercised in the comparative interpretation of tests applied to apparently healthy animals as different tests may vary in their sensitivities relative to stage of incubation and pathogenesis of the disease.

Brief details of the five tests now approved for use in the European Union are provided below. See section Section B.1. regarding the interchangeability of terminology for the abnormal form of PrP.

- The Enfer Test (Enfer test distributed by: Abbott Diagnostics, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, IL 60034-3500, USA) is a qualitative microplate-based chemiluminescent immunassay for the detection of resistant prion protein (PrP^{res}). PrP^{res} in extracted samples is bound to prepared wells in microtitre plates and detected with an anti-PrP polyclonal primary antibody, a horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate.

- The Platelia® or TeSeE® test (Platelia Bio-Rad (A subsequent version of this test the TeSeE test has been approved by the EU and is now available): Bio-Rad, 3 Boulevard Raymond Poincar, 92430 Marnes la Coquette, Paris, France) is a sandwich immunoassay which uses two monoclonals for the detection of abnormal prion protein, which is resistant to proteinase K. The presence of abnormal PrP is indicated by a coloured substrate. The test has been approved in a manual and automated format.

- The Prionics® Check Western Blot (Prionics Check Western Blot and Prionics LIA: Prionics, Wagistrasse 27a, CH-8952 Schlieren, Switzerland, Distributer: Roche Diagnostics GmbH, 68298 Mannheim, Germany) is a Western blot based method for the detection of the disease specific form of the prion protein. Samples are homogenised and treated with protease to remove normal PrP (PrP^{c}) and to convert PrP^{res} to the PrP27-30 fragment. Samples are separated by gel electrophoresis, transferred to a membrane and PrP27-30 is identified by its immuno-reactivity with anti-PrP antibodies and by the

molecular weights of stained bands.

- The CDI test (CDI test: InPro Biotechnology, 870 Dubuque Avenue, South San Francisco, CA 94080, USA) is an automated conformational dependent immunoassay for the detection of disease causing PrP^{res}. The test is based on a sandwich ELISA using a europium labelled antibody to detect any PrP present in the sample. The comparison of normal (PrP^{c}) to total (PrP^{c} and PrP^{res}) allows an assessment to be made of the level of abnormal PrP^{res} in the sample.

- The Prionics® Check LIA (Prionics LIA, Wagistrasse 27a, CH-8952 Schlieren, Switzerland, www.prionics.com) is a microplate-based luminescence immunoassay. Samples are treated with proteinase K to degrade PrP^{c}, while PrP^{res} is reduced to the 27–30 kd fragment. The proteolytic reaction is stopped and PrP^{res} contained in the sample is detected in a sandwich immunoassay with a chemiluminescent enzyme substrate reaction.

d) Other diagnostic tests

The demonstration of characteristic fibrils, the bovine counterpart of SAF (see Chapter 2.4.8. Scrapie), by negative-stain electron microscopy in detergent extracts of fresh or frozen brain or spinal cord tissue (75, 79, 92) has been used as an additional diagnostic method for BSE and may be particularly useful when histopathological approaches are precluded by the occurrence of post-mortem decomposition (74). Recent work on scrapie indicates that, with modification, the method may be applied successfully to formalin-fixed tissue (13). Detection of fibrils has been shown to correlate well with the histopathological diagnosis of BSE (77), but does not offer the specificity or sensitivity available from IHC or immunoblotting methods. Some of the rapid immunological tests are also effective in the presence of autolysis (14) and given their greater sensitivity than SAF detection, may be the preferred tests in such circumstances.

BSE infection can be shown by intracerebral/intraperitoneal inoculation (39) or by feeding mice with brain tissue from terminally affected cattle (6), but bioassay is impractical for routine diagnosis because of the long incubation period (greater than or equal to 292 days). Further development of transgenic mice overexpressing the bovine PrP gene may potentially offer bioassays with reduced incubation periods for BSE. However, data obtained from one such study did not

derive incubation periods substantially shorter than that of conventional mouse strains (12).

There remains the need for a test for BSE that can be applied to the live animal and has sensitivity capable of detecting PrP^{res} at the low levels, such as may occur in the early stages of incubation of the disease. Potential approaches to diagnosis are published from time to time, often of a preliminary nature and based on limited amounts of data. None has progressed to the point of peer review and evaluation by others, and claims should be interpreted with care.

Certain protein markers, notably apolipoprotein E (Apo E), can be detected by two-dimensional gel electrophoresis in cerebrospinal fluid of clinically suspected, histopathologically confirmed cases of BSE (53). Apo E is, however, is a nonspecific marker for neurodegeneration and has not been shown to be useful for diagnosis of preclinical cases of BSE. Assay of cerebrospinal fluid for the 14-3-3 protein is not of diagnostic use in BSE (7068). Similarly, studies of S-100 proteins in cerebrospinal fluid (45) and serum (67) did not give results that would provide diagnostically useful tests for BSE. The electrochemical detection of metabolites in urine (52) has a final performance validation that gave specificity and sensitivity values below that required for it to have a possible independent role in the diagnosis of BSE. Preliminary data indicating that a derivative of the PrP molecule may be detected in the urine of infected cattle (77) is leading to further investigations with a view to the marketing of a diagnostic test kit, but further evaluation and peer review of the data are required.

2. Serological tests

Similarities with scrapie, in which no immune response in the host has been detected, suggest that there is not likely to be an immune response in BSE.

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.

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NB: There are OIE Reference Laboratories for Bovine spongiform encephalopathy (please consult the OIE Web site at: http://www.oie.int/eng/OIE/organisation/en_LR.htm).

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