Project title:	**Bioassay of BSE infectivity in neural and non-neural tissues by intracerebral inoculation of cattle**
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Contractor:	Veterinary Laboratories Agency Woodham Lane New Haw Addlestone Surrey KT15 3NB
Project leader:	S.A.C. Hawkins (direct line: 01932 357818) e-mail: s.a.c.Hawkins@vla.defra.gsi.gov.uk
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EXECUTIVE SUMMARY

Bovine spongiform encephalopathy (BSE) is a priondisease of domestic cattle that was first recognised in Great Britain in 1986. This epidemic is thought to have originated by contamination of commercially processed feed with a scrapie-like agent.

A control strategy for BSE was constructed on assumptions about the development of disease (pathogenesis) based on natural scrapie of sheep and a scrapie origin of BSE. Control measures included the removal of prescribed offals from all slaughtered cattle to prevent contamination of human or animal food. However mouse bioassay failed to detect infectivity in tissues of the lymphoretiicular system of naturally-infected cattle questioning the hypothesis of a close similarity between the pathogenesis of BSE and natural scrapie.

Uniformity of the pathology of BSE provided the first indication that the BSE epidemic involved a single strain of a scrapie-like pathogen. Subsequently this was supported by the uniformity of incubation periods in cattle injected with BSE and the consistency of the data on primary transmissions to mice. This uniformity provided the necessary predictability required for an experimental study of the pathogenesis of BSE in the natural host

A study (SE1901/MO3011) was initiated in 1992 in order to determine the development of infectivity and pathology following oral exposure of calves to affected cattle brain homogenate. Challenged and control cattle were sequentially killed and a large range of tissues and body fluids were collected to a range of protocols for mouse bioassay for detection of infectivity, histopathology and detection of PrP^{D}

Infectivity was detected in the distal ileum, central nervous system (brain and spinal cord), sensory ganglia (cervical dorsal root ganglia, thoracic dorsal root ganglia and trigeminal ganglia) and, at a single timepoint, bone marrow. No infectivity was demonstrated in lymphoreticular tissues, apart from the involvement of Peyer's patches which are concentrated in the distal ileum; peripheral nervous system; other areas of the alimentary tract, striated muscle and major viscera. One reason for this may be limitations of the sensitivity of the assay.

A further experiment has investigated the underestimation of the titre of infectivity of BSE tissue when titrated across a species barrier in mice and found this to a factor of 500 fold. Therefore, to improve the sensitivity of the assay of tissues from the experimental oral exposure study, additional assays of selected tissues have been conducted by intracerebral inoculation of cattle. These assays provide an assessment of infectivity in a tissue utilizing the same host species, by the most efficient route of inoculation

Calves were sourced from herds free of clinical BSE and with a history of no exposure to meat and bone meal. Inocula were freshly prepared from frozen tissue samples and comprised tissue pools from challenged cattle killed at sequential selected timepoints of the oral exposure study. Groups of five calves were inoculated intracerebrally at 4-6 months of age with 1ml of inoculum, using a semi-stereotaxic technique which ensured anatomically reproducible deposition of inoculum in the brain. After inoculation experimental groups were housed separately with husbandry procedures in place to ensure that the integrity of each group was maintained.

On confirmation of clinical signs of BSE and at the termination of the study cattle were killed and the brain removed and examined by histopathology, immunohistochemistry for PrP^{D} , Western blot analysis and BioRad Elisa.

Following scientific review of the end point it was considered appropriate to terminate the study with cull of the remaining animals from 84 months post challenge.

Infectivity was confirmed in pooled central nervous system tissues from cattle killed 32 months following oral exposure to BSE and from distal ileum sampled from cattle 6, 10 and 18 months after oral exposure. In each of these groups 5/5 inoculated cattle succumbed to disease. These results are consistent with previous results of mouse bioassay of these tissues.

In addition, infectivity was detected in pooled palatine tonsil tissue from cattle killed 10 months after oral exposure with 1/5 cattle in the challenge group affected and in a pool of lymphoid tissue from the nictitating membrane collected from 10 natural field cases of BSE with, again, 1/5 cattle in the challenge group affected. These results raised the likelihood that bovines had a wider lymphoreticular pathogenesis.

However, there is no evidence, to date, from studies of the pathogenesis of BSE that there is, at any stage of the disease, either widespread lymphatic or haematogenous spread of agent. With the development and application of more sensitive methods of detection of agent and PrP^{D} in future, further evidence of the distribution of agent in BSE infected cattle may emerge, but the present findings reinforce the notion that the involvement of the lymphoreticular system in BSE is highly restricted.

GLOSSARY

ADAS – Agricultural Development Advisory Service
BSE – Bovine spongiform encephalopathy
C - Cervical
CNS – Central nervous system
DNA – Deoxyribonucleic acid
EDTA – Ethylenediamine-tetraacetic acid
Elisa – Enzyme linked immuno-sorbent assay
HE – Haematoxylin and Eosin
IHC - Immunohistochemistry
L - Lumbar
LN – Lymph node
LRS – Lymphoreticular system
Mab – Monoclonal antibody
MBM – Meat and bone meal
PCR – Polymerase chain reaction
p.i. – post infection
PrP – Prion protein
PrP^{D} – Disease specific prion protein
SRM – Specified risk materials
T - Thoracic
TSE – Transmissible spongiform encephalopathy
UK – United Kingdom
WB – Western blotting

AIMS AND OBJECTIVES

To determine, qualitatively, infectivity in certain tissues, including specified risk materials (SRM), of cattle infected orally with 100g BSE-affected brain and killed at specified times after exposure, using a within species bioassay. This provides the most sensitive means of detection of infectivity in the tissues in order to identify public health risks from exposure to bovine tissues. Bioassay of BSE or a related disease is necessary because there is currently no other method for conclusively demonstrating the infectious pathogen.
The results indicate qualitative differences in the use of cattle rather than mice for bioassay of BSE infectivity in experimentally, orally infected cattle. They also provide information for use in quantitative analysis of risks of human exposure to infected bovine tissues and the necessity to retain restrictions on SRM entering the human food chain.

EXPERIMENTAL PROCEDURES

Selection and tagging of cattle

For the assay of infectivity by intracerebral inoculation of cattle, two-week-old Friesian/Holstein calves were assembled from UK herds free of clinical BSE and with a history of no exposure to meat and bone meal (MBM). On arrival the ear tag number of each calf was recorded. A further laboratory reference was assigned by use of an additional ear tag, readable at a short distance from the animal.

PrP genotyping

The PrP genotype of the calves, with respect to the octarepeat polymorphism (Goldmann and others 1991), was determined from EDTA blood samples, to ensure allocation of representative genotypes among experimental groups, as far as was possible given that the acquisition of calves for the programme of inoculations was over a three year period (1996-1999). DNA was extracted directly from the blood samples, and a 400-bp fragment of the PrP gene, containing the octarepeat region, was amplified by the polymerase chain reaction (PCR) method and the octarepeat number detected by the length of the amplimer generated by gel electrophoresis. This polymorphism has not been associated with any differential susceptibility to BSE

infection (Hunter and others 1994). Only 6:6 and 6:5 genotypes were detected among the total of 325 calves in the study.

Preparation of inocula

Inocula were freshly prepared from frozen tissues samples and comprised tissue pools from exposed cattle killed at selected sequential kill time points of an oral exposure study (Wells and others 1996) or from confirmed cases of natural BSE (Table 1). To prepare inocula, 2g of tissue (comprising equal amounts of the identical tissue in the case of the challenged animals of each time point) were macerated and ground in a minimum volume of sterile saline, diluted to give a 10% suspension in saline, and finally passed through a gauze filter. The inocula were stored as 5ml aliquots at -70ºC. Ampicillin (5mg per aliquot) was added to suspensions prepared from distal ileum and tonsil.

Intracerebral inoculation of calves

Groups of five calves aged 4-6 months were inoculated intracerebrally with 1.0ml of inoculum, using a semi-stereotaxic technique (Wells and Hawkins 2004) which ensured anatomically reproducible deposition of inoculum in the brain. Following induction of anaesthesia intubation, for maintenance of closed circuit gaseous anaesthesia, was carried out and the animal was positioned in sternal recumbency. A formula for determining reproducibility of the inoculation site was devised by a series of measurements conducted post mortem on the sagitally cut head of calves. It was proposed that the inoculum should be injected deep into the brain stem (mesencephalon) and along the needle tract passing through medial parietal cerebral cortex, ensuring installation in multiple neuroanatomical locations. The trephine was paramedian in the mid-frontal region of the head, at a rostro-caudal level that minimized the depth of the frontal sinus, with the needle inserted at such an angle to the midline such that it would traverse the midline ventral to the dorsal sagittal sinus. The trephine was made at 90º to the rostro-caudal slope of the frontal region of the head and inclined 25º laterally, by rotating and counter rotating the trephine instrument while applying pressure, until a hole was made through the skull. A spinal hypodermic needle (Yale 9cm) was inserted to a calculated depth and the inoculum was deposited along the entire length of the needle tract whilst the needle was slowly withdrawn. Two groups of five calves were similarly inoculated with sterile saline to serve as procedural controls.

Husbandry and clinical monitoring

Experimental groups were housed separately in purpose built accommodation avoiding nose-to-nose contact and preventing possible contact with excreta between groups. Husbandry procedures ensured maintenance of the integrity of each group by the rigorous use of detailed protocols and dedicated equipment and protective clothing. Cattle were monitored clinically for signs of disease onset. Passive observations, during daily husbandry routines and weekly visits by veterinary staff, were made in order to detect behavioural changes and signs associated with the transmissible spongiform encephalopathies (TSE). These features of the clinical presentation are well documented (Austin and others 1994, Konold and others 2004). Responses to handling and restraint were assessed during routine weighing and foot trimming. Weekly observations to determine the onset , extent and progression of clinical signs were undertaken. The animal's activity when the pen was first approached, it's reaction to the observer's movements and the interaction between the animals in the group were recorded. A detailed neurological examination was also carried out every 3-4 months and prior to the cull of an animal.

On development of clinical signs, indicative of BSE, cattle were killed by intravenous injection of pentobarbitone sodium and exsanguinated.

Post-mortem diagnosis

Detection of abnormal PrP in BSE-affected cattle brain first confirmed BSE as a prion disease of cattle (Hope et al., 1988). Therefore, caudal brain stem was taken fresh for detection of PrP using Prionics Check Western immunoblotting method probed with Mab 6H4 (Cooley and others 2001, Stack 2004) and a commercially available Elisa method (BioRad TeSeE) approved for the diagnosis of BSE (Grassi and others 2001, Deslys and others 2001). Brain, spinal cord, medial retropharyngeal lymph node, palatine tonsil, mesenteric lymph node and distal ileum were removed, fixed in 10% formol saline (brain and spinal cord) or phosphate-buffered, neutral, 10% formalin (viscera) and then processed routinely through to paraffin wax. Sections of the brain (medulla at the level of the obex, medulla at the level of the caudal cerebellar peduncles, mesencephalon at the level of the inferior colliculi, mesencephalon at the level of the superior colliculi, cerebellum, parietal cortex, occipital cortex and frontal cortex) were prepared and stained with haematoxylin and

eosin (HE) and examined for evidence of vacuolar changes. Immunohistochemical examination for disease specific PrP, utilizing the R145 monoclonal antibody (Terry and others 2003), was applied to the medulla at the level of the obex, medulla at the level of the caudal cerebellar peduncles, mesencephalon at the level of the inferior colliculi, mesencephalon at the level of the superior colliculi, medial retropharyngeal lymph node, palatine tonsil, mesenteric lymph node and distal ileum. All sections were examined by light microscopy. The presence of abnormal PrP was confirmed when the configurations and distribution of immunostaining were consistent with those previously described in BSE (Wells and others 1994, Wells and Wilesmith 1995). Any animal that had to be killed due to intercurrent disease (Table 2), welfare reasons or at the termination of the study were examined similarly.

Project termination

In advance of the project review milestone the contractor was commissioned to undertake a review of the project and its predecessors in order to draw up a scientific rationale for determining the period post-inoculation for culling the surviving cattle. The analyses indicated that a termination of the studies at approximately 7 years post inoculation (p.i.) would not result in any measurable loss of data on the infectivity of tissues under assay. A cull of the animals on the studies was therefore initiated with post mortem collection of tissues as detailed above.

RESULTS

The status of the assays by intracerebral inoculation of cattle is given in Table 3.

Consistent with previous results of the bioassay of tissues from the sequential kill experimental oral exposure study (Wells and others 1998), assay by intracerebral inoculation of cattle confirmed the presence of infectivity in a pool of caudal medulla and spinal cord taken from cattle 32 months after oral exposure to the BSE agent and in distal ileum from cattle 6, 10 and 18 months after exposure (see Table 3). In each of these groups 5/5 inoculated cattle succumbed to disease. The mean incubation periods recorded for the distal ileum groups were 27, 22 and 24 months respectively. This is consistent with the RIII mouse bioassay which indicated a rising titre (reducing mean incubation) of infectivity in distal ileum from cattle six months to

14 months after exposure and a plateau of incubation period in mice inoculated with distal ileum from cattle 18 months after exposure (Wells and others 1996; 1998).

One of the five cattle injected intracerebrally with pooled palatine tonsil from the three cattle killed 10 months post-exposure from the oral exposure study was confirmed to show clinically progressive signs of BSE at 45 months post-inoculation. A diagnosis of BSE was confirmed by histopathological examination, immunohistochemistry, Western blotting and BioRad Elisa (Wells and others 2005). The remaining four animals in this group and cattle inoculated intracerebrally with palatine tonsil from other kill time points in the experimental oral exposure study were all negative for BSE by the same four tests when culled due to intercurrent disease or at the end of the study.

In addition, infectivity was detected in a pool of lymphoid tissue from the nictitating membrane collected from 10 natural field cases of BSE with 1/5 animals in this challenge group showing clinically progressive signs of BSE at 33 months post inoculation. Histopathological examination, immunohistochemistry, Western blotting and BioRad Elisa confirmed the diagnosis.

All of the remaining assays of tissues by intracerebral inoculation of cattle from the oral exposure study proved negative.

DISCUSSION

Assay by intracerebral inoculation of cattle confirmed the presence of infectivity in a central nervous system (CNS) tissues taken from cattle killed 32 months after exposure to the BSE agent and in distal ileum from cattle killed 6, 10 and 18 months after exposure. The detection of infectivity in palatine tonsil and lymphoid tissue of the nictitating membrane by intracerebral challenge of cattle is the first evidence of detection of infectivity in these tissues and has raised the likelihood that bovines have a wider lymphoreticular pathogenesis. However, there is no evidence, to date, from studies of the pathogenesis of BSE that there is, at any stage of the disease, either widespread lymphatic or haematogenous spread of agent. With the development and application of more sensitive methods of detection of agent and disease specific PrP in future, further evidence of the distribution of agent in BSE

infected cattle may emerge, but the present findings reinforce the notion that the involvement of the lymphoreticular system in BSE is highly restricted.

No apparent effect on susceptibility to experimental infection was observed with respect to differences in the PrP gene octarepeat polymorphism among the intracerebrally inoculated cattle succumbing to disease. The single animals which developed disease in the groups inoculated with palatine tonsil and nictitating membrane were a 6:6 genotype. Among the cattle developing the disease the ratio of 6:6 to 6:5 genotypes was closely similar to that reported in cattle naturally infected with BSE (Hunter and others 1994).

Studies of infectivity in tonsil of naturally occurring cases of BSE (Fraser and Foster 1994) and of experimentally orally infected cattle, when assayed in conventional mouse strains (Wells and others 1998, EC 2002), have not detected agent. Immunohistochemical examination of palatine tonsil from cattle at all sequential time points throughout the experimental oral exposure study revealed no evidence of disease specific immunostaining. Review of the mouse bioassay of the palatine tonsil from cattle killed at the 10 month post challenge time point of the oral exposure study by applying immunohistochemistry for detection of disease specific PrP in mouse brains which had been diagnosed negative on histopathological examination (Wells and others 1999) confirmed a negative result.

There have been no previous transmission studies using lymphoid tissue of the nictitating membrane however immunohistochemistry has detected PrP in this tissue in scrapie affected sheep and clinically normal flockmates (Kim and others 2001) and indeed biopsy of this tissue is used as a screen for the presence of scrapie infection.

In studies of this nature the potential for such findings to be the result of experimental error or artifact has to be considered. The detection of infectivity in the tonsil is unlikely to be due to the persistence of inoculum due to its entrapment after dosing in the experimental oral exposure study, because of the lack of evidence for this phenomenon in relation to earlier timepoints in the study at this and other anatomical sites where residues of inoculum could occur and the absence of any evidence of PrP immunostaining on examination by immunohistochemistry. Contamination of the source tissues at necropsy is also unlikely due to the undetectable levels in all other tissues, except for distal ileum, at the 10 month post-exposure time point of the oral

exposure study. Infection of the animals on farm prior to sourcing for the study is also unlikely as no cases of BSE have been traced before or since on the source farms.

The observation of infectivity in tonsil and lymphoid tissue of the nictitating membrane is not without precedent as it is a feature of naturally occurring scrapie of sheep. In contrast to BSE in cattle, scrapie (or experimental BSE) infection of lymphoid tissues of sheep throughout much of the incubation period is well recognized.

The association of infectivity in BSE infected cattle with both distal ileal Peyer's Patches (Terry and others 2003) and, as reported here, palatine tonsil has pathogenetic importance. Both tissues have a role in the sampling of antigen from the lumen of the digestive system and the presence of infectivity at these sites from early in the incubation period suggests that the early events in the pathogenesis of BSE and scrapie are quite similar. The major difference in the lymphoreticular system (LRS) involvement between the two diseases appears to be host determined, quantitative differences in the levels of PrP that accumulate.

Development and application of more sensitive methods of detection of agent and PrP in the future may demonstrate further evidence of the distribution of agent in BSE infected cattle, but the present findings reinforce the notion that the involvement of the LRS in BSE of cattle is highly restricted.

TABLE 1: Tissues assayed by intracerebral challenge of cattle

Tissue	Time of death of orally exposed cattle (months)							
	6	10	18	22	26	32	36	Natural BSE
Caudal medulla/spinal cord (pooled)				√	√	√		
Caudal medulla	√	√	√		√			
Spinal cord[1]	√	√	√		√			
Skeletal muscle[2]	√		√		√	√		
Sciatic/radial nerves (pooled)	√		√		√	√		
Parotid/submandibular salivary gland			√		√			
Distal ileum	√	√	√		√	√		
Liver	√		√		√	√		
Spleen	√	√	√		√			
Thymus	√	√						
Tonsil	√	√	√		√			
Mesenteric lymph node	√		√		√			
Muscle lymph nodes[3]	√		√		√			
Buffy coat	√		√		√	√		
Bone marrow				√	√	√	√	
Skin			√		√	√		
Kidney	√		√		√	√		
Urine			√					
Nictitating membrane								√

[1]Pool of spinal cord levels C_2-C_3, T_{10}-T_{11}, L_3-L_4

[2]Pool of masseter/semitendinosus/longissimus dorsi

[3]Pool of superficial cervical/popliteal lymph nodes

TABLE 2: Details of intercurrent losses

Tissue/timepoint	Animal ref	Time p.i animal killed (months)	Reason for early cull
Muscle (18m p.i.)	CL672	26	Tibial fracture
Muscle (18m p.i.)	CL675	68	Found recumbent/unable to rise – history of joint problems (osteoarthritis)
Muscle (18m p.i.)	CL677	95	Found recumbent/unable to rise – severe bruising of thoracic spinal area at PM
Peripheral nerve (32m p.i.)	CL727	70	Found dead
Peripheral nerve (32m p.i.)	CL729	93	Progressive weight loss/loss of condition – lung tumour at PM
Liver (18m p.i.)	CL683	80	Spastic syndrome
Kidney (18m p.i.)	CL713	93	Chronic lameness – degenerative joint disease
Kidney (18m p.i.)	CL705	96	Chronic lameness – arthritis/osteochondrosis
Saline control	CL673	97	Chronic lameness – degenerative joint disease
Buffy coat (32m p.i.)	CL723	74	Anal constriction resulting in chronic constipation
Saline control	CN983	31	Found in lateral recumbency and bloated – no gross abnormalities at PM
Thymus (6m p.i.)	CN990	67	Urolithiasis
Skin (32m p.i.)	CN1023	83	Urolithiasis
Spinal cord (10m p.i.)	CN1049	63	Urolithiasis
Peripheral nerve (6m p.i.)	CN1261	64	Spastic syndrome
Mesenteric LN (6m p.i.)	CN1378	81	Urolithiasis
Skin (18m p.i.)	CP1470	76	Recumbency following a spinal injury
Tonsil (18m p.i.)	CP1500	56	Urolithiasis
Spinal cord (18m p.i.)	CP1518	36	Found dead
Muscle (26m p.i.)	CP1538	80	Chronic lameness - osteochondrosis
Muscle LN (26m p.i.)	CP1544	50	Found recumbent/unable to rise
Buffy coat (26m p.i.)	CP1613	77	Urolithiasis
Salivary gland (26m p.i.)	CP1608	54	Sudden death
Skin (26m p.i.)	CP1627	81	Rumen impaction
Spleen (26m p.i.)	CP1642	69	Chronic lameness/recumbency

Tonsil (26m p.i.)	CP1622	86	Lordosis/stiff gait – fusion of lumbar vertebrae
Spinal cord (26m p.i.)	CP1639	83	Chronic lameness - osteochondrosis
Spinal cord (26m p.i.)	CP1653	83	Chronic lameness - osteochondrosis
Bone marrow (32m p.i.)	CP1591	79	Urolithiasis
Bone marrow (32m p.i.)	CP1651	80	Urolithiasis
Bone marrow (26m p.i.)	CP1696	70	Spastic syndrome
Urine (18m p.i.)	CP1857	78	Rumen impaction and internal bleeding
Nictitating membrane (field case)	CR2021	53	Found recumbent/unable to rise

TABLE 3: Results of histopathological examination, immunohistochemistry (IHC), Western blotting (WB) and Biorad Elisa

Tissue/timepoint	Animal ref	Months p.i	Histopathology	IHC	WB	Biorad
Kidney (32m p.i.)	CL714	97	-ve	-ve	-ve	-ve
	CL715	97	-ve	-ve	-ve	-ve
	CL719	97	-ve	-ve	-ve	-ve
	CL722	97	-ve	-ve	-ve	-ve
	CM748	97	-ve	-ve	-ve	-ve
Muscle (18m p.i.)	CL672	26	-ve	-ve	-ve	-ve
	CL675	68	-ve	-ve	-ve	-ve
	CL677	95	-ve	-ve	-ve	-ve
	CL 681	98	-ve	-ve	-ve	-ve
	CL682	98	-ve	-ve	-ve	-ve
Peripheral nerve (32m p.i.)	CL716	96	-ve	-ve	-ve	-ve
	CL717	96	-ve	-ve	-ve	-ve
	CL727	70	-ve	-ve	-ve	-ve
	CL729	93	-ve	-ve	-ve	-ve
	CL738	96	-ve	-ve	-ve	-ve
Liver (18m p.i.)	CL678	98	-ve	-ve	-ve	-ve
	CL680	98	-ve	-ve	-ve	-ve
	CL683	80	-ve	-ve	-ve	-ve
	CL700	98	-ve	-ve	-ve	-ve
	CL707	98	-ve	-ve	-ve	-ve
Liver (32m p.i.)	CL710	98	-ve	-ve	-ve	-ve
	CL718	98	-ve	-ve	-ve	-ve
	CL732	98	-ve	-ve	-ve	-ve
	CL734	98	-ve	-ve	-ve	-ve
	CL735	98	-ve	-ve	-ve	-ve
Kidney (18m p.i.)	CL701	98	-ve	-ve	-ve	-ve
	CL704	98	-ve	-ve	-ve	-ve
	CL705	96	-ve	-ve	-ve	-ve
	CL706	98	-ve	-ve	-ve	-ve
	CL713	93	-ve	-ve	-ve	-ve
Distal ileum (18m p.i.)	CL702	24	+ve	+ve	+ve	+ve
	CL703	24	+ve	+ve	+ve	+ve
	CL708	25	+ve	+ve	+ve	+ve
	CL724	25	+ve	+ve	+ve	+ve
	CL725	24	+ve	+ve	+ve	+ve
Saline controls	CL673	97	-ve	-ve	-ve	-ve
	CL674	99	-ve	-ve	-ve	-ve
	CL676	99	-ve	-ve	-ve	-ve
	CL679	99	-ve	-ve	-ve	-ve
	CL684	99	-ve	-ve	-ve	-ve
Buffy coat (32m p.i.)	CL712	98	-ve	-ve	-ve	-ve
	CL723	74	-ve	-ve	-ve	-ve
	CL728	98	-ve	-ve	-ve	-ve
	CL736	98	-ve	-ve	-ve	-ve
	CL737	98	-ve	-ve	-ve	-ve

Muscle (32m p.i.)	CL720	99	-ve	-ve	-ve	-ve
	CL731	99	-ve	-ve	-ve	-ve
	CL733	99	-ve	-ve	-ve	-ve
	CL711	99	-ve	-ve	-ve	-ve
	CL721	99	-ve	-ve	-ve	-ve
Saline controls	CN971	85	-ve	-ve	-ve	-ve
	CN973	85	-ve	-ve	-ve	-ve
	CN977	85	-ve	-ve	-ve	-ve
	CN980	85	-ve	-ve	-ve	-ve
	CN983	31	-ve	-ve	-ve	-ve
CNS – caudal medulla/sp. cord (32m p.i.)	CN972	22	+ve	+ve	+ve	+ve
	CN974	24	+ve	+ve	+ve	+ve
	CN975	24	+ve	+ve	+ve	+ve
	CN978	23	+ve	+ve	+ve	+ve
	CN979	23	+ve	+ve	+ve	+ve
Distal ileum (32m p.i.)	CM857	86	-ve	-ve	-ve	-ve
	CN984	86	-ve	-ve	-ve	-ve
	CN991	86	-ve	-ve	-ve	-ve
	CN992	86	-ve	-ve	-ve	-ve
	CN995	86	-ve	-ve	-ve	-ve
CNS - caudal medulla/sp.cord (22m p.i.)	CM860	86	-ve	-ve	-ve	-ve
	CN985	86	-ve	-ve	-ve	-ve
	CN986	86	-ve	-ve	-ve	-ve
	CN988	86	-ve	-ve	-ve	-ve
	CN989	86	-ve	-ve	-ve	-ve
Thymus (6m p.i.)	CN987	84	-ve	-ve	-ve	-ve
	CN990	67	-ve	-ve	-ve	-ve
	CN993	84	-ve	-ve	-ve	-ve
	CN1027	84	-ve	-ve	-ve	-ve
	CN1029	84	-ve	-ve	-ve	-ve
Distal ileum (10m p.i.)	CN1019	22	+ve	+ve	+ve	+ve
	CN1020	22	+ve	+ve	+ve	+ve
	CN1021	23	+ve	+ve	+ve	+ve
	CN1026	22	+ve	+ve	+ve	+ve
	CN1030	23	+ve	+ve	+ve	+ve
Skin (32m p.i.)	CN1022	84	-ve	-ve	-ve	-ve
	CN1023	83	-ve	-ve	-ve	-ve
	CN1024	84	-ve	-ve	-ve	-ve
	CN1025	84	-ve	-ve	-ve	-ve
	CN1028	84	-ve	-ve	-ve	-ve
Caudal medulla (10m p.i.)	CN1035	84	-ve	-ve	-ve	-ve
	CN1037	84	-ve	-ve	-ve	-ve
	CN1039	84	-ve	-ve	-ve	-ve
	CN1045	84	-ve	-ve	-ve	-ve
	CN1051	84	-ve	-ve	-ve	-ve

Pooled CNS (caudal medulla, C2-3, T10-11) (26m p.i.)	CN1036	85	-ve	-ve	-ve	-ve
	CN1040	85	-ve	-ve	-ve	-ve
	CN1042	85	-ve	-ve	-ve	-ve
	CN1046	85	-ve	-ve	-ve	-ve
	CN1047	85	-ve	-ve	-ve	-ve
Spinal cord (10m p.i.)	CN1043	84	-ve	-ve	-ve	-ve
	CN1044	84	-ve	-ve	-ve	-ve
	CN1048	84	-ve	-ve	-ve	-ve
	CN1049	63	-ve	-ve	-ve	-ve
	CN1086	84	-ve	-ve	-ve	-ve
Spleen (10m p.i.)	CN1085	84	-ve	-ve	-ve	-ve
	CN1088	84	-ve	-ve	-ve	-ve
	CN1089	84	-ve	-ve	-ve	-ve
	CN1090	84	-ve	-ve	-ve	-ve
	CN1091	84	-ve	-ve	-ve	-ve
Tonsil (10m p.i.)	CN1147	84	-ve	-ve	-ve	-ve
	CN1149	84	-ve	-ve	-ve	-ve
	CN1150	45	+ve	+ve	+ve	+ve
	CN1151	84	-ve	-ve	-ve	-ve
	CN1161	84	-ve	-ve	-ve	-ve
Thymus (10m p.i.)	CN1152	84	-ve	-ve	-ve	-ve
	CN1153	84	-ve	-ve	-ve	-ve
	CN1158	84	-ve	-ve	-ve	-ve
	CN1162	84	-ve	-ve	-ve	-ve
	CN1164	84	-ve	-ve	-ve	-ve
Kidney (6m p.i.)	CN1155	84	-ve	-ve	-ve	-ve
	CN1156	84	-ve	-ve	-ve	-ve
	CN1159	84	-ve	-ve	-ve	-ve
	CN1163	84	-ve	-ve	-ve	-ve
	CN1165	84	-ve	-ve	-ve	-ve
Liver (6m p.i.)	CN1203	84	-ve	-ve	-ve	-ve
	CN1205	84	-ve	-ve	-ve	-ve
	CN1206	84	-ve	-ve	-ve	-ve
	CN1209	84	-ve	-ve	-ve	-ve
	CN1212	84	-ve	-ve	-ve	-ve
Muscle (6m p.i.)	CN1148	84	-ve	-ve	-ve	-ve
	CN1204	84	-ve	-ve	-ve	-ve
	CN1207	84	-ve	-ve	-ve	-ve
	CN1208	84	-ve	-ve	-ve	-ve
	CN1211	84	-ve	-ve	-ve	-ve
Muscle lymph nodes (6m p.i.)	CN1210	84	-ve	-ve	-ve	-ve
	CN1213	84	-ve	-ve	-ve	-ve
	CN1247	84	-ve	-ve	-ve	-ve
	CN1257	84	-ve	-ve	-ve	-ve
	CN1303	84	-ve	-ve	-ve	-ve

Peripheral nerve (6m p.i.)	CN1251	84	-ve	-ve	-ve	-ve
	CN1252	84	-ve	-ve	-ve	-ve
	CN1253	84	-ve	-ve	-ve	-ve
	CN1261	64	-ve	-ve	-ve	-ve
	CN1264	84	-ve	-ve	-ve	-ve
Buffy coat (6m p.i.)	CN1248	85	-ve	-ve	-ve	-ve
	CN1250	85	-ve	-ve	-ve	-ve
	CN1254	85	-ve	-ve	-ve	-ve
	CN1259	85	-ve	-ve	-ve	-ve
	CN1263	85	-ve	-ve	-ve	-ve
Spleen (6m p.i.)	CN1297	85	-ve	-ve	-ve	-ve
	CN1298	85	-ve	-ve	-ve	-ve
	CN1306	85	-ve	-ve	-ve	-ve
	CN1312	85	-ve	-ve	-ve	-ve
	CN1313	85	-ve	-ve	-ve	-ve
Tonsil (6m p.i.)	CN1255	85	-ve	-ve	-ve	-ve
	CN1300	85	-ve	-ve	-ve	-ve
	CN1310	85	-ve	-ve	-ve	-ve
	CP1746	86	-ve	-ve	-ve	-ve
	CP1747	86	-ve	-ve	-ve	-ve
Distal ileum (6m p.i.)	CN1260	25	+ve	+ve	+ve	+ve
	CN1324	26	+ve	+ve	+ve	+ve
	CN1372	30	+ve	+ve	+ve	+ve
	CN1375	27	+ve	+ve	+ve	+ve
	CN1376	29	+ve	+ve	+ve	+ve
Mesenteric lymph nodes (6m p.i.)	CN1262	85	-ve	-ve	-ve	-ve
	CN1307	85	-ve	-ve	-ve	-ve
	CN1311	85	-ve	-ve	-ve	-ve
	CN1377	85	-ve	-ve	-ve	-ve
	CN1378	81	-ve	-ve	-ve	-ve
Caudal medulla (6m p.i.)	CN1308	86	-ve	-ve	-ve	-ve
	CN1374	86	-ve	-ve	-ve	-ve
	CN1395	86	-ve	-ve	-ve	-ve
	CN1401	86	-ve	-ve	-ve	-ve
	CP1501	86	-ve	-ve	-ve	-ve
Spinal cord (6m p.i.)	CN1323	86	-ve	-ve	-ve	-ve
	CN1392	86	-ve	-ve	-ve	-ve
	CN1399	86	-ve	-ve	-ve	-ve
	CN1407	86	-ve	-ve	-ve	-ve
	CN1408	86	-ve	-ve	-ve	-ve
Peripheral nerve (18m p.i.)	CN1391	86	-ve	-ve	-ve	-ve
	CN1393	86	-ve	-ve	-ve	-ve
	CN1409	86	-ve	-ve	-ve	-ve
	CN1410	86	-ve	-ve	-ve	-ve
	CN1411	86	-ve	-ve	-ve	-ve
Buffy coat (18m p.i.)	CN1373	87	-ve	-ve	-ve	-ve
	CN1397	87	-ve	-ve	-ve	-ve
	CN1403	87	-ve	-ve	-ve	-ve
	CN1412	87	-ve	-ve	-ve	-ve
	CN1415	87	-ve	-ve	-ve	-ve

Muscle LNs (18m p.i.)	CN1398	87	-ve	-ve	-ve	-ve
	CN1400	87	-ve	-ve	-ve	-ve
	CN1402	87	-ve	-ve	-ve	-ve
	CN1413	87	-ve	-ve	-ve	-ve
	CN1414	87	-ve	-ve	-ve	-ve
Salivary gland (18m p.i.)	CN1406	87	-ve	-ve	-ve	-ve
	CP1462	87	-ve	-ve	-ve	-ve
	CP1465	87	-ve	-ve	-ve	-ve
	CP1466	87	-ve	-ve	-ve	-ve
	CP1467	87	-ve	-ve	-ve	-ve
Skin (18m p.i.)	CN1405	87	-ve	-ve	-ve	-ve
	CP1468	87	-ve	-ve	-ve	-ve
	CP1469	87	-ve	-ve	-ve	-ve
	CP1470	76	-ve	-ve	-ve	-ve
	CP1471	87	-ve	-ve	-ve	-ve
Mesenteric LN (18m p.i.)	CP1473	87	-ve	-ve	-ve	-ve
	CP1494	87	-ve	-ve	-ve	-ve
	CP1495	87	-ve	-ve	-ve	-ve
	CP1497	87	-ve	-ve	-ve	-ve
	CP1498	87	-ve	-ve	-ve	-ve
Spleen (18m p.i.)	CP1472	87	-ve	-ve	-ve	-ve
	CP1499	87	-ve	-ve	-ve	-ve
	CP1503	87	-ve	-ve	-ve	-ve
	CP1504	87	-ve	-ve	-ve	-ve
	CP1505	87	-ve	-ve	-ve	-ve
Tonsil (18m p.i)	CP1463	88	-ve	-ve	-ve	-ve
	CP1464	88	-ve	-ve	-ve	-ve
	CP1500	56	-ve	-ve	-ve	-ve
	CP1513	88	-ve	-ve	-ve	-ve
	CP1516	88	-ve	-ve	-ve	-ve
Caudal medulla (18m p.i.)	CP1496	88	-ve	-ve	-ve	-ve
	CP1512	88	-ve	-ve	-ve	-ve
	CP1514	87	-ve	-ve	-ve	-ve
	CP1515	88	-ve	-ve	-ve	-ve
	CP1517	88	-ve	-ve	-ve	-ve
Spinal cord (18m p.i.)	CP1518	36	-ve	-ve	-ve	-ve
	CP1519	88	-ve	-ve	-ve	-ve
	CP1521	88	-ve	-ve	-ve	-ve
	CP1536	88	-ve	-ve	-ve	-ve
	CP1520	88	-ve	-ve	-ve	-ve
Skeletal muscle (26m p.i.)	CP1523	88	-ve	-ve	-ve	-ve
	CP1524	88	-ve	-ve	-ve	-ve
	CP1538	80	-ve	-ve	-ve	-ve
	CP1540	88	-ve	-ve	-ve	-ve
	CP1541	88	-ve	-ve	-ve	-ve
Muscle lymph nodes (26m p.i.	CP1522	88	-ve	-ve	-ve	-ve
	CP1525	88	-ve	-ve	-ve	-ve
	CP1543	88	-ve	-ve	-ve	-ve
	CP1544	50	-ve	-ve	-ve	-ve
	CP1546	88	-ve	-ve	-ve	-ve

Liver (26m p.i.)	CP1533	89	-ve	-ve	-ve	-ve
	CP1535	89	-ve	-ve	-ve	-ve
	CP1537	89	-ve	-ve	-ve	-ve
	CP1542	89	-ve	-ve	-ve	-ve
	CP1545	89	-ve	-ve	-ve	-ve
Kidney (26m p.i.)	CP1530	89	-ve	-ve	-ve	-ve
	CP1532	89	-ve	-ve	-ve	-ve
	CP1581	89	-ve	-ve	-ve	-ve
	CP1580	89	-ve	-ve	-ve	-ve
	CP1702	86	-ve	-ve	-ve	-ve
Distal ileum (26m p.i.)	CP1603	89	-ve	-ve	-ve	-ve
	CP1548	89	-ve	-ve	-ve	-ve
	CP1600	89	-ve	-ve	-ve	-ve
	CP1601	89	-ve	-ve	-ve	-ve
	CP1604	88	-ve	-ve	-ve	-ve
Peripheral nerve (26m p.i.)	CP1527	89	-ve	-ve	-ve	-ve
	CP1529	89	-ve	-ve	-ve	-ve
	CP1609	89	-ve	-ve	-ve	-ve
	CP1610	89	-ve	-ve	-ve	-ve
	CP1611	89	-ve	-ve	-ve	-ve
Buffy coat (26m p.i.)	CP1582	89	-ve	-ve	-ve	-ve
	CP1605	89	-ve	-ve	-ve	-ve
	CP1606	89	-ve	-ve	-ve	-ve
	CP1612	89	-ve	-ve	-ve	-ve
	CP1613	77	-ve	-ve	-ve	-ve
Salivary gland (26m p.i.)	CP1526	89	-ve	-ve	-ve	-ve
	CP1607	89	-ve	-ve	-ve	-ve
	CP1608	54	-ve	-ve	-ve	-ve
	CP1614	89	-ve	-ve	-ve	-ve
	CP1630	89	-ve	-ve	-ve	-ve
Skin (26m p.i.)	CP1620	89	-ve	-ve	-ve	-ve
	CP1623	89	-ve	-ve	-ve	-ve
	CP1626	89	-ve	-ve	-ve	-ve
	CP1627	81	-ve	-ve	-ve	-ve
	CP1638	89	-ve	-ve	-ve	-ve
Mesenteric lymph nodes (26m p.i.)	CP1624	89	-ve	-ve	-ve	-ve
	CP1625	89	-ve	-ve	-ve	-ve
	CP1628	89	-ve	-ve	-ve	-ve
	CP1636	89	-ve	-ve	-ve	-ve
	CP1640	89	-ve	-ve	-ve	-ve
Spleen (26m p.i.)	CP1642	69	-ve	-ve	-ve	-ve
	CP1643	89	-ve	-ve	-ve	-ve
	CP1656	89	-ve	-ve	-ve	-ve
	CP1658	89	-ve	-ve	-ve	-ve
	CP1660	89	-ve	-ve	-ve	-ve
Tonsil (26m p.i.)	CP1594	90	-ve	-ve	-ve	-ve
	CP1595	90	-ve	-ve	-ve	-ve
	CP1622	86	-ve	-ve	-ve	-ve
	CP1632	90	-ve	-ve	-ve	-ve
	CP1637	90	-ve	-ve	-ve	-ve

Caudal medulla (26m p.i.)	CP1645	90	-ve	-ve	-ve	-ve
	CP1647	90	-ve	-ve	-ve	-ve
	CP1650	90	-ve	-ve	-ve	-ve
	CP1654	90	-ve	-ve	-ve	-ve
	CR2016	84	-ve	-ve	-ve	-ve
Spinal cord (26m p.i.)	CP1629	90	-ve	-ve	-ve	-ve
	CP1631	90	-ve	-ve	-ve	-ve
	CP1639	83	-ve	-ve	-ve	-ve
	CP1653	83	-ve	-ve	-ve	-ve
	CP1770	90	-ve	-ve	-ve	-ve
Bone marrow (32m p.i.)	CP1591	79	-ve	-ve	-ve	-ve
	CP1592	88	-ve	-ve	-ve	-ve
	CP1602	91	-ve	-ve	-ve	-ve
	CP1644	91	-ve	-ve	-ve	-ve
	CP1651	80	-ve	-ve	-ve	-ve
Bone marrow (36m p.i.)	CP1689	90	-ve	-ve	-ve	-ve
	CP1691	90	-ve	-ve	-ve	-ve
	CP1692	90	-ve	-ve	-ve	-ve
	CP1700	90	-ve	-ve	-ve	-ve
	CP1701	90	-ve	-ve	-ve	-ve
Bone marrow (22m p.i.)	CP1641	91	-ve	-ve	-ve	-ve
	CP1681	91	-ve	-ve	-ve	-ve
	CP1682	91	-ve	-ve	-ve	-ve
	CP1683	91	-ve	-ve	-ve	-ve
	CP1685	91	-ve	-ve	-ve	-ve
Bone marrow (26m p.i.)	CP1690	87	-ve	-ve	-ve	-ve
	CP1694	91	-ve	-ve	-ve	-ve
	CP1696	70	-ve	-ve	-ve	-ve
	CP1739	91	-ve	-ve	-ve	-ve
	CP1741	91	-ve	-ve	-ve	-ve
Urine (18m p.i.)	CP1803	87	-ve	-ve	-ve	-ve
	CP1853	87	-ve	-ve	-ve	-ve
	CP1856	87	-ve	-ve	-ve	-ve
	CP1857	78	-ve	-ve	-ve	-ve
	CP1862	86	-ve	-ve	-ve	-ve
Nictitating membrane (field case)	CR2010	84	-ve	-ve	-ve	-ve
	CR2011	33	+ve	+ve	+ve	+ve
	CR2015	84	-ve	-ve	-ve	-ve
	CR2018	84	-ve	-ve	-ve	-ve
	CR2021	53	-ve	-ve	-ve	-ve

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REFERENCES

Austin, A.R., Hawkins, S.A.C., Kelay, N.S. and Simmons, M.M. (1994) New observations on the clinical signs of BSE and scrapie. In : Bradley, R., Marchant, B. eds. Transmissible spongiform encephalopathies. Proceedings of a Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels, 14-15th September 1993. Kluwer Academics, Dordrecht. pp277-284

Cooley, W.A., Clark, J.K., Ryder, S.J., Davis, L.A., Farrelly, S.S.J. and Stack, M.J. (2001) Evaluation of a rapid Western immunoblotting procedure for the diagnosis of bovine spongiform encephalopathy. Journal of Comparative Pathology **125**, 64-70

Deslys, J.P., Comoy, E., Hawkins, S.A.C., Simon, S., Schimmel, H., Wells, G.A.H., Grassi, J. and Moynagh, J. (2001) Screening slaughtered cattle for BSE. Nature **409**, 476-478

European Commission (2002) SSC of 8th November 2002. Update of the opinion on TSE infectivity distribution in ruminant tissues. Initially adopted by the Scientific Steering Committee at its meeting of 10-11 January 2002 and amended at its meeting of 7-8 November 2002, following submission of (1) a risk assessment by the German Federal Ministry of Consumer Protection, Food and Agriculture and (2) new scientific evidence regarding BSE infectivity distribution in tonsils.

Fraser, H. and Foster, J. (1994) Transmission to mice, sheep and goats and bioassay of bovine tissues. In : Bradley, R., Marchant, B. eds. Transmissible spongiform encephalopathies. Proceedings of a Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels, 14-15th September 1993. Kluwer Academics, Dordrecht. pp 145-159

Goldmann, W., Hunter, N., Martin, T., Dawson, M. and Hope, J. (1991) Different forms of bovine PrP gene have five or six copies of a short, G-C rich element within the protein coding exon. Journal of General Virology **72**, 201-204

Grassi, J., Comoy, E., Simon, S., Créminon, C., Frobert, Y., Trapmann, S., Schimmel, H., Hawkins, S.A.C., Moynagh, J., Deslys, J.P. and Wells, G.A.H. (2001) Rapid test for the preclinical postmortem diagnosis of BSE in central nervous system tissue. Veterinary Record **149**, 577-582

Hope, J., Reekie, L.J., Hunter, N., Multhaup, G., Beyreuther, K., White, H., Scott, A.C., Stack, M.J., Dawson, M., & Wells, G.A. (1988) Fibrils from brains of cows with new cattle disease contain scrapie-associated protein *Nature 336*, 390-2.

Hunter, N., Goldmann, W., Smith, G. and Hope, J. (1994) Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. Veterinary Record **135**, 400-403

Kim, H., O'Rourke, K.I., Walter, M., Purchase, H.G., Enck, J. and Shin, T.K. 2001 Immunohistochemical detection of scrapie prion proteins in clinically normal sheep. Journal of Veterinary Diagnostic Investigation **13**, 89-91

Konold, T., Bone, G., Ryder, S., Hawkins, S.A.C., Courtin, F. and Berthelin-Baker, C. (2004) Clinical findings in 78 suspected cases of bovine spongiform encephalopathy in Great Britain. Veterinary Record **155**, 659-666

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*, Chapter 8, pp. 97-116. Edited by S. Lehmann & J. Grassi. Berlin: Birkhäuser Verlag.

Terry, L.A., Marsh, S., Ryder, S.J., Hawkins, S.A.C., Wells, G.A.H. and Spencer, Y.I. (2003) Detection of disease-specific PrP in the distal ileum of cattle orally exposed to the BSE agent. Veterinary Record **152**, 387-392

Wells, G.A.H., Dawson, M., Hawkins, S.A.C., Austin, A.R., Green, R.B., Dexter, I., Horigan, M.W., and Simmons, M.M. (1996) Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy. In: Bovine Spongiform Encephalopathy: The BSE Dilemma. Ed C.J.Gibbs Jr. Springer-Verlag. pp 28-44

Wells, G.A.H. and Hawkins, S.A.C. (2004) Animal models of transmissible spongiform encephalopathies: experimental infection, observation and tissue collection. In: Methods and Tools in Biosciences and Medicine. Techniques in Prion Research, Ed S.Lehmann and J. Grassi, Birkhauser Verlag, Basel. pp 37-71

Wells, G.A.H., Hawkins, S.A.C., Green, R.B., Austin, A.R., Dexter, I, Spencer, Y.I., Chaplin, M.J. and Dawson, M. (1998)Preliminary observations on the pathogenesis

of experimental bovine spongiform encephalopathy (BSE): an update. Veterinary Record **142**, 103-106

Wells, G.A.H., Hawkins, S.A.C., Green, R.B., Spencer, Y.I., Dexter, I and Dawson, M. (1999) Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). Veterinary Record **144**, 292-294

Wells, G.A.H., Spiropoulos, J., Hawkins, S.A.C. and Ryder, S.J. (2005) Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivty in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. Veterinary Record **156**, 401-407

Wells, G.A.H. and Wilesmith, J.W. (1995) The neuropathology and epidemiology of bovine spongiform encephalopathy. Brain Pathology **5**, 91-103