

# Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs

Gerald A. H. Wells,<sup>1</sup> Stephen A. C. Hawkins,<sup>1</sup> Anthony R. Austin,<sup>2</sup> Stephen J. Ryder,<sup>1</sup> Stanley H. Done,<sup>1</sup> Robert B. Green,<sup>1</sup> Ian Dexter,<sup>1</sup> Michael Dawson<sup>3</sup> and Richard H. Kimberlin<sup>4</sup>

## Correspondence

Gerald Wells

g.a.h.wells@vla.defra.gsi.gov.uk

<sup>1</sup>Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK

<sup>2</sup>FARA, Oak Farm, Harpsden Bottom, Henley-on-Thames, Oxon RG9 4HY, UK

<sup>3</sup>NSPAC, DEFRA, Whittington Road, Worcester WR5 2SU, UK

<sup>4</sup>SARDAS, 27 Laverockdale Park, Edinburgh EH13 0QE, UK

Studies to test the transmissibility of the bovine spongiform encephalopathy (BSE) agent to pigs began in 1989. Parenteral inoculation of the agent by three routes simultaneously (intracranially, intravenously and intraperitoneally) produced disease with an incubation period range of 69–150 weeks. Pre-clinical pathological changes were detected in two pigs killed electively at 105 and 106 weeks post-inoculation. Infectivity was detected by bioassay in inbred mice in the CNS of those pigs that developed spongiform encephalopathy. Infectivity was also found in the stomach, jejunum, distal ileum and pancreas of terminally affected pigs. These findings show that pigs are susceptible to BSE. In contrast, disease failed to occur in pigs retained for 7 years after exposure by feeding BSE-affected brain on three separate days, at 1–2 week intervals. The amounts fed each day were equivalent to the maximum daily intake of meat and bone meal in rations for pigs aged 8 weeks. No infectivity was found in tissues assayed from the pigs exposed orally. This included tissues of the alimentary tract. It is suggested that these pigs did not become infected. The relatively high oral exposure used in these experiments compared with feed-borne exposure in the field may explain the absence of an epidemic of spongiform encephalopathy in domestic pigs concurrent with the BSE epidemic in the UK.

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## INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a scrapie-like, transmissible spongiform encephalopathy (TSE) of domestic cattle, which was first recognized in the UK in 1986 (Wells *et al.*, 1987). It presented as an extended common source epidemic with the major route of transmission via feedstuffs containing meat and bone meal (MBM) contaminated with a scrapie-like pathogen (Wilesmith *et al.*, 1988; Wilesmith, 1991, 1998). That other species, including pigs, had been exposed to ruminant-derived MBM suggested the potential for wider infection with this agent. At the initiation in 1987–1988 of a research programme to determine the transmissibility of BSE to a variety of possible host species, there was no information on the susceptibility of the pig to TSE infection, either from experimental or natural exposure, apart from an unsuccessful attempt to transmit kuru (Gibbs *et al.*, 1979).

A prerequisite to testing the response of the pig to oral exposure was to test the susceptibility to BSE infection by

the most effective routes of exposure. Preliminary results of the experimental transmission of BSE to the domestic pig after simultaneous parenteral inoculations by three routes with brain homogenate from BSE-infected cows have been reported previously (Dawson *et al.*, 1990b, 1991, 1994). Subsequently, inoculation of mice with brain material from this successful transmission showed that the agent in the initial affected pig shared common biological characteristics with the agent transmitted to mice from naturally occurring cases of BSE (Bruce *et al.*, 1994). After the establishment of BSE infection in the pig by injection, a study was initiated to examine whether BSE could be transmitted to pigs by dietary exposure (Dawson *et al.*, 1991).

Here we report the completed findings of these parenteral [(intracranial (i.c.), intravenous (i.v.) and intraperitoneal (i.p.)] and oral transmission studies, including the results of the bioassay for infectivity in mice of a range of tissues sampled at necropsy from exposed and control pigs.

## METHODS

The protocols for these studies have been given in brief outline previously (Dawson *et al.*, 1990b, 1991).

**Inocula.** For the parenteral study, the inoculum was an unclarified 10% (w/v) saline suspension of a pool of homogenized brain stem from four natural, histopathologically confirmed cases of BSE in unrelated Holstein–Friesian dairy cattle (BSE 1–4). The source material was identical to that used for the preparation of inocula for primary parenteral transmissions of the BSE agent to mice (Fraser *et al.*, 1988, 1992; Bruce *et al.*, 1994) and to cattle (Dawson *et al.*, 1990a, 1991, 1994). Primary transmissions from the brain stem material of each of the four BSE cases gave similar incubation periods in inbred strains of RIII or C57BL mice.

End-point titration of brain-stem material from one case (BSE 1) in each mouse strain indicated  $10^{5.1}$  to  $10^{5.2}$  i.c. mouse ID<sub>50</sub> units g<sup>-1</sup> (Fraser *et al.*, 1992). For the dietary exposure, a pool of homogenized brain (brain pool code BBP 5/90) was prepared from 29 natural, histopathologically confirmed cases of BSE killed in 1990. After brain removal, a transverse block of medulla oblongata was taken from each brain for histopathological diagnosis of BSE and the remaining brain tissue homogenized progressively until a puree of all brains was achieved. This was aliquoted into new 1 litre containers and frozen awaiting administration to the pigs.

Two separate primary transmissions of a 1:10 dilution of this brain pool in RIII mice produced disease in 14/15 and 15/15 mice with mean incubation periods of 415 (SEM ± 12.3) days and 403 (SEM ± 9.0) days. Primary transmissions of the same brain pool in C57BL/6 mice produced disease in 13/16 mice with a mean incubation period of 776 (SEM ± 27.8) days (unpublished observations). An end-point titration of infectivity in the pooled brain in RIII mice gave a value of  $10^{2.4}$  i.c./i.p. mouse LD<sub>50</sub> units (g tissue)<sup>-1</sup>.

**Animals and exposures.** A total of 21 Landrace crossed Large White piglets from three litters of the former Central Veterinary Laboratory's specific pathogen-free breeding herd were used in the parenteral exposure study. Piglets were allocated at random to exposed and control groups. All piglets were weaned and at 1–2 weeks of age (February–March 1989) were inoculated in batches by three routes simultaneously whilst under halothane anaesthesia. All male piglets were castrated whilst anaesthetized. The 10 exposure group piglets received the brain stem inoculum (BSE 1–4) and the 11 control piglets received normal saline. Eleven controls were used because one piglet died 10 days post-inoculation (p.i.) and was replaced at the time of inoculation of the last batch of control piglets. Each piglet was injected with 0.5 ml intracranially into the left cerebral hemisphere by percutaneous, transcalvareal injection, 1–2 ml intravenously into the left cranial vena cava and 8–9 ml intraperitoneally through the abdominal wall adjacent to the umbilicus. The i.c. injection site was located 1 cm lateral to the midline in the frontal region, equidistant from the lateral commissure of the eye and the base of the cranial border of the pinna. The trephine was made with a 16- or 18-gauge hypodermic needle and the inoculation made through a 25-gauge hypodermic needle. The use of multiple routes of inoculation was to maximize the chance of infection, as it is known that in transmission of some scrapie isolates, the i.c. route fails to establish infection directly in the brain but some of the inoculum infects peripherally (Kimberlin, 1993a). Exposed and control pigs were housed separately and penned in groups of two to three. Pigs from the breeding herd had been fed commercial rations containing MBM, providing the potential for their exposure to the BSE agent. The piglets were also reared on a commercial ration for pigs.

For the dietary exposure study, recipient pigs representative of popular breed types in the UK were purchased. Five pregnant sows of different

breed types were obtained from different commercial sources. The piglets from each of the litters produced were fed a ration free of MBM. After weaning at 7–8 weeks, exposed and control groups of 10 piglets were assembled by assigning a castrated male and a female from each of the five litters to each group. The exposure was calculated with the purpose of replacing a maximum daily intake of MBM for the age of pig under commercial dietary conditions with a dry matter equivalent of the BSE-infected brain pool homogenate (BBP 5/90). The calculation assumed that, on average, a commercially reared piglet would be fed a total of 56 kg of concentrated rations during the first 3 months (12 weeks) after weaning. Therefore, the average daily intake would be 677 g of feed and assuming a water content of 20%, the dry matter fed per day would be 534 g. The maximum inclusion of MBM in commercial rations was assumed to be 15%, which gives a figure of 80 g per day. To replace this amount of MBM with an equivalent dry matter content of brain (approximately 20%) required the daily feeding of 400 g of brain homogenate. Accordingly, in May–June 1990 when the pigs were 8 weeks of age, approximately 4 kg of the homogenate was fed to the exposed group on three successive occasions, at 1–2 week intervals. On each occasion, 4 kg of the thawed brain homogenate was deposited on the floor of the pen of the exposure group, and the pigs were closely observed to confirm that each consumed a substantial portion of the homogenate prior to receiving the normal ration. Assuming that equal amounts were ingested, the total amount of infected brain tissue to which each pig was exposed was therefore 1.2 kg. Control pigs received only the normal ration, free of MBM, and were housed apart from the exposed group.

**Post-inoculation protocol and necropsy sampling.** Pigs were kept under daily observation by animal technicians, and regular veterinary clinical assessments were made one to four times weekly, depending on the stage of the studies. On the development and progression of clinical signs suggesting an encephalopathy or the occurrence of intercurrent life-threatening or welfare-compromising disease, individual animals were sedated with azaperone (Stresnil; Janssen Animal Health) and killed by the intravenous injection of pentobarbitone sodium followed by exsanguination. In the absence of clinical disease at 2 years p.i., it was planned to kill half of the surviving exposed and control groups at that point and the remaining animals at 5 years p.i. The 2-year elective kills were to afford an opportunity of obtaining evidence of disease at an earlier stage in the studies than provided by the terminal kill times. This was reviewed in the course of the dietary exposure study at 4 years p.i., and the surviving animals were kept until 7 years p.i. to improve detection of cases of disease with potentially longer incubations.

At necropsy, a range of tissues was sampled aseptically, stored at -70 °C and subsequently specific tissues were selected for bioassay of infectivity in mice (see Tables 3 and 4). A segment of spinal cord (C<sub>1-2</sub>) was collected and stored at -70 °C for examination for scrapie-associated fibrils (SAF) (Stack *et al.*, 1996). Tissues were also collected into 10% formal saline (CNS and dorsal root ganglia) or phosphate-buffered, neutral 10% formalin (BF) (viscera, lymphoreticular tissues, endocrine organs, peripheral nervous system and skeletal muscles) for histopathological examinations. Eyes were fixed in Zenker's fluid. CNS tissues were routinely processed to paraffin wax and histological sections were cut at 5 µm thickness and stained with haematoxylin and eosin. Anti-PrP immunohistochemistry was conducted on sections of brain at five levels, as previously described (Ryder *et al.*, 2000). Whereas in the parenteral inoculation study the brains from all pigs were examined by this method, in the oral exposure study the brains of all exposed pigs but only those controls surviving to 359–371 weeks p.i. were examined. Remaining neural and non-neural tissues from these animals were also routinely processed and sections prepared for histopathological examination.

**Mouse bioassay of infectivity.** Inocula were prepared either from single tissues or from single-tissue pools of pigs from exposed or control groups of the parenteral inoculation study and the oral exposure study. Tissues were selected according to the outcome of each experiment (see Tables 3 and 4). The method of inoculum preparation has been published in detail previously (Wells *et al.*, 1996). Test and control inocula were each assayed in C57BL/6 (*Sinc*<sup>87</sup>) or RIII (*Sinc*<sup>87</sup>) inbred mice according to standard procedures (Bruce *et al.*, 1994). Each inoculum was assayed in 20 mice. Each mouse was injected with 0.02 ml intracranially and 0.1 ml intraperitoneally of a 10% suspension of tissue in saline. The limit of detectability of BSE agent by titration of primary isolates in RIII mice has been calculated as  $10^{1.4}$  mouse i.c./i.p. ID<sub>50</sub> units (g tissue)<sup>-1</sup> (Kimberlin, 1996) and no differences between titre end-points by i.c. inoculation of BSE agent have been found between RIII and C57BL mice (Fraser *et al.*, 1992). Detailed clinical monitoring was carried out from 250 days p.i. and the mice were scored for signs of neurological disease. The clinical end-point, when mice show clear signs of neurological disease, was determined according to established criteria for calculating incubation period (Dickinson *et al.*, 1968). At necropsy, the brain of each mouse was removed, fixed in BF and processed routinely for histopathological examination. Any mice surviving to the end-point for incubation and expression of disease (700 or 950 days for C57BL mice; 650 days for RIII mice; see Tables 3 and 4) were killed and examined similarly, as were any mice that died or were killed *in extremis*.

Following the histopathological assessment of mice inoculated with tissues from pigs exposed to the BSE agent, immunohistochemical examination for the presence of disease-specific PrP was made on the brains of all mice in certain selected tissue groups. This extension of the standard protocol for assessment of bioassay results was used, as described previously (Wells *et al.*, 1999), as it has the potential to provide improved specificity and sensitivity of detection of disease in experimental transmissions of scrapie and related agents. The justification for the selective use of this method in these studies was twofold: first, to confirm observations of evidence of transmission in the parenteral inoculation study and to clarify the disease status of mice surviving in groups where the tissue had been shown to contain infectivity; and secondly, to seek further evidence of possible infectivity in the oral exposure study in tissues corresponding to those that had evidence of infectivity in the parenteral inoculation study. The immunohistochemical method used was essentially as previously applied (Wells *et al.*, 1999), but the antiserum to PrP was 486, a polyclonal rabbit serum (similar to 971), prepared at the Veterinary Laboratories Agency (R. Jackman, unpublished data) to a synthetic peptide equivalent to bovine residues 230–244. The serum has been trialled previously to confirm its ability to demonstrate disease-specific PrP immunostaining in mouse brain (data not shown). Serum was applied at a dilution of 1:2500 using an avidin–biotin–peroxidase complex technique. Normal rabbit serum was used as a control.

## RESULTS

### Transmission of BSE to pigs by parenteral inoculations

Details of animal identifications, the periods between inoculation and euthanasia, and the experimental outcome for each pig are given in Table 1.

Two control and two exposed pigs died or were killed as a result of intercurrent disease early in the study. Transmission of BSE was established in one exposed pig killed 74 weeks p.i. – the first case of an experimentally induced

TSE in the pig (Dawson *et al.*, 1990b). Histopathological evidence of transmission was found next in two out of three clinically normal exposed pigs (36/91, 38/91), which were killed along with four control pigs, as scheduled, at 105–107 weeks p.i. (Dawson *et al.*, 1994; Hawkins *et al.*, 1998). The four remaining exposed pigs developed clinical neurological signs and were killed between 139 and 163 weeks p.i. Clinical signs in the pigs killed at 139–163 weeks p.i. were similar to those reported in the initial affected pig (58/90), killed at 74 weeks p.i. (Dawson *et al.*, 1990b). The progress of the behavioural changes followed a generally similar pattern in all five clinically affected pigs, beginning with intermittent inappetance, apparent agonistic reactions to attendants and mild ataxia. Later, behaviour suggested apparent confusion, and there was progressive locomotor disability with weakness and adventitious movement disorders, particularly tremor. Finally, there was persistent recumbency with difficulty in rising.

The period between inoculation and the development of unequivocal progressive neurological signs, when the pigs were killed, was from 74 to 163 weeks. Estimates of incubation periods, based on the time between inoculation and the onset of the earliest clinical signs, could be made in retrospect from the pattern of progress of the clinical signs. The earliest evidence of clinical signs ranged from 5 weeks (58/90) to 13 weeks (310/92) prior to euthanasia, giving an estimated incubation period range of 69–150 weeks.

Neuropathological changes indicative of spongiform encephalopathy were evident in all of the pigs that developed progressive neurological signs and in two of the three clinically normal exposed pigs killed electively at 105–107 weeks p.i. Vacuolar changes were severe and extended throughout most brain regions in clinically affected pigs killed 139–163 weeks p.i., whereas in the two pre-clinically affected pigs (36/91, 38/91), killed at 105 and 106 weeks p.i., the lesions were variable in extent and appreciably less severe. The changes in pig 58/90, with the shortest incubation period, were intermediate in severity between these two groups. In all five clinically affected pigs, the vacuolar changes were most concentrated in the basal nuclei and diminished in intensity caudally, with the least severe changes in the caudal brainstem. The forms and patterns of PrP deposition in the CNS of the pigs with spongiform encephalopathy, as visualized by immunohistochemistry, was typical of disease-specific PrP accumulations in other species. Details of the neuropathology of the pigs in this experiment have been reported previously (Ryder *et al.*, 2000). Histopathological examinations of between 19 and 32 non-neural and peripheral nervous system tissues from all pigs revealed only minor background and incidental pathology for the species.

Samples from all pigs in the study were examined for SAF. In only one animal with spongiform encephalopathy (58/90; killed 74 weeks p.i.) were SAF detected (Dawson *et al.*, 1990b).

**Table 1.** Animal identification, period between inoculation and euthanasia and experimental outcome of pigs in parenteral exposure study

Animal identification	Time of death p.i. (weeks)	Experimental outcome
<b>Exposed</b>		
28/90	47	Intercurrent deaths
34/90	50	
58/90	74	Neurological signs/spongiform encephalopathy/PrP*
36/91	105	Clinically normal/electively killed (2 years p.i.) /spongiform encephalopathy/PrP
38/91	106	
42/91	107	Clinically normal/electively killed/NSE†/no PrP
341/91	139	Neurological signs/spongiform encephalopathy/PrP
42/92	151	
66/92	152	
310/92	163	
<b>Controls</b>		
PD119	1	Intercurrent deaths
23/89	3	
33/91	105	Clinically normal/electively killed (2 years p.i.)/NSE/no PrP
37/91	105	
44/91	105	
40/91	106	
129/92	153	Clinically normal/electively killed control for exposed affected pigs (139–163 weeks p.i.)/NSE/no PrP
193/94	255	Clinically normal/electively killed (7 years p.i.)/NSE/no PrP
402/94	259	
425/94	263	
433/94	263	

\*PrP detected by immunohistochemistry.

†NSE, No spongiform encephalopathy.

### Survival of pigs exposed to the BSE agent by feeding

Details of animal identifications, the periods between the first of the three exposures and euthanasia, and the experimental outcome for each pig are given in Table 2.

One exposed pig (380/91) was killed at 80 weeks p.i. and one control pig (148/92) was killed at 92 weeks p.i. because of a progressive lameness in both experimental groups. Remedial measures included treatment with a non-steroidal anti-inflammatory preparation, increasing bedding materials and changes in the mineral constituents of the ration. In addition to clinically normal exposed and control pigs killed, as planned, 103–109 weeks p.i., further unscheduled euthanasia of pigs included one (1482/93) at 173 weeks p.i. because of a slowly progressive ataxia with hind limb hyperflexion and another (2172/95) at 265 weeks p.i. because of recent loss of body weight, anorexia and polydipsia. This latter animal had a clinical history of hermaphroditism and post-mortem examination also revealed a chronic pyelo-nephritis and cystitis. Also, one control pig (214/97) rapidly lost body weight and was killed at 350 weeks p.i. Post-mortem examination revealed mild,

localized, non-suppurative meningo-chorio-encephalitis. The remaining three exposed and four control pigs were killed at 359–371 weeks p.i. No clinical signs suggestive of a response to exposure to BSE were evident in these remaining pigs. Examination of the brains and spinal cords revealed no significant histopathological changes suggestive of transmission. However, both exposed and control pigs in this experiment had varying degrees of neuropil vacuolation of the rostral colliculus and sometimes also of the hypothalamus. No evidence of disease-specific patterns of PrP immunostaining was detected. The histopathological examination of non-neural and peripheral nervous system tissues from challenged and control pigs revealed only minor background and incidental pathology for the species.

### Mouse bioassay of infectivity

Tissue pools or individual tissues were selected for bioassay according to the outcome of each experiment and the requirement for efficient use of assay mice (Tables 3 and 4).

In the study of parenteral exposure, infectivity was demonstrated in the CNS of the animals with clinical signs and spongiform encephalopathy and the two animals that were

**Table 2.** Animal identification, period between the first of three exposures and euthanasia, and experimental outcome of pigs in the oral exposure study

Animal identification	Weeks p.i.	Experimental outcome
<b>Exposed</b>		
380/91	80	Intercurrent death (lameness)/NSE*
400/92	104	Clinically normal†/electively killed (2 years p.i.)/NSE
406/92	105	
580/92	108	
618/92	109	
1482/93	173	Intercurrent disease/killed with ataxia/NSE
2172/95	265	Intercurrent disease/killed with loss of body weight, anorexia and polydipsia in association with hermaphroditism/NSE
1014/97	366	Clinically normal†, electively killed (7 years p.i.)/NSE
1020/97	367	
1029/97	367	
<b>Controls</b>		
148/92	92	Intercurrent death (lameness)/NSE
364/92	103	Clinically normal†/electively killed (2 years p.i.)/NSE
373/92	104	
533/92	108	
569/92	108	
214/97	350	Intercurrent disease/killed with rapid loss of body weight/encephalitis/NSE
560/97	359	Clinically normal†/electively killed (7 years p.i.)/NSE
1190/97	370	
1194/97	370	
1303/97	371	

\*NSE, No spongiform encephalopathy.

†Clinically normal apart from progressive lameness, which affected some pigs from both exposed and control groups.

asymptomatic but had spongiform encephalopathy. Infectivity was also detected in certain of the alimentary tissues pooled from pigs killed 139–163 weeks p.i. with clinical signs and spongiform encephalopathy (Table 5). When immunohistochemistry was applied to the brains of mice from these assays, an increased incidence of positive mice was shown for most of the CNS tissue and each of the four alimentary tissues. Immunohistochemical examination of brains of bioassay mice inoculated with brain or spinal cord from the exposed pig killed at 107 weeks, which had no evidence of disease, proved negative. Bioassay of all other tissues from exposed and control animals in the parenteral study (Table 3) did not reveal evidence of infectivity. The mean survival times of C57BL/6 mice for all negative assays of different tissues ranged from 645 (SEM = 56.1) to 826 (SEM = 27.9) days. All but six mice throughout the assays (maximum two in any one tissue group) were available for histopathological assessment of the brain.

After exposure by feeding, bioassays of neural and non-neural tissue pools from pigs killed at 80–109 weeks and from pigs killed at 350–371 weeks (Table 4) did not detect infectivity. Application of PrP immunohistochemical examination to brains of bioassay mice inoculated with

brain (cerebral cortex and medulla), spinal cord, spleen, mesenteric lymph node, stomach, pancreas or distal ileum, pooled from pigs killed 350–371 weeks after exposure, proved negative. The mean survival times of C57BL/6 mice for different tissues ranged from 614 (SEM = 34.2) to 698 (SEM = 5.7) days. The corresponding mean survival times of RIII mice were 475 (SEM = 45.9) to 635 (SEM = 15.1) days. All but six mice from each of the mouse strains throughout the assays (maximum two in any one tissue group) were available for histopathological assessment of the brain.

## DISCUSSION

BSE has been transmitted experimentally by parenteral routes from cattle to mice (Fraser *et al.*, 1988, 1992), cattle (Dawson *et al.*, 1990a), sheep and goats (Foster *et al.*, 1993; Fraser & Foster, 1994), mink (*Mustela vison*) (Robinson *et al.*, 1994), marmoset (*Callithrix jacchus*) (Baker *et al.*, 1993), cynomolgus macaque (Lasmézas *et al.*, 1996) and guinea pig (M. Dawson, P. Griffiths & S. J. Ryder, unpublished data). The present study shows that BSE is transmissible from cattle to pigs that were exposed parenterally.

**Table 3.** Tissues inoculated into C57BL/6 mice (assay end-point 950 days p.i.) from the parenteral exposure study

Inocula were composed of single tissues from individual animals or single-tissue pools from multiple animals of exposed or control groups as indicated for each of the time-points or ranges after exposure at which assays were conducted.

Tissue	Time after exposure when killed (weeks)			
	74*	105–107†	139–163‡	255–263§
Brain (frontal cerebral cortex)	•	•	•	•
Spinal cord (C <sub>2-3</sub> )		•		•
Spleen	•		•	
Thymus			•	
Mesenteric LN	•		•	
Stomach			•	
Jejunum			•	
Distal ileum			•	
Pancreas			•	
Liver	•		•	
Kidney	•		•	

\*A single clinically affected histopathologically confirmed exposed pig (58/90).

†Two clinically normal exposed pigs (36/91, 38/91) with spongiform encephalopathy, one clinically normal exposed pig with no significant brain lesions (42/91) and four age-matched normal control pigs (33/91, 37/91, 40/91, 44/91); tissues from each animal were assayed individually.

‡Four clinically affected, histopathologically confirmed pigs (341/91, 42/92, 66/92, 310/92); each tissue was assayed as a pool from all affected pigs. Tissues from a clinically normal control pig (129/92) were also assayed.

§Four clinically normal control pigs (193/94, 402/94, 425/94 and 433/94); tissues from each animal were assayed individually.

The two asymptomatic cases (36/91, 38/91) were killed within the estimated incubation period range of 69–150 weeks and their pathology was less severe than that of all of the clinical cases (Ryder *et al.*, 2000). This is consistent with a pre-clinical stage of disease, since in a variety of other animal models of TSE, the earliest neuropathological changes occur before the onset of clinical signs. The reasons for the wide variation in incubation period in the pig after parenteral exposure to BSE are unclear, although such variations are not uncommon in the interspecies transmission of TSE agents.

The species barrier to the transmission of TSE agents is determined by several factors of which two of the most important are the strain of agent and genetic differences between the donor and recipient species, particularly with respect to the PrP gene (Kimberlin, 1990; Bruce *et al.*, 1994). However, variation in the BSE agent is unlikely to explain the range of incubation periods observed in pigs. Repeated isolations of the BSE agent from cattle have provided evidence that it is a single major strain of TSE agent, which has remained stable in cattle through the course of the epidemic in the UK (Bruce *et al.*, 1994). This conclusion is supported by the unchanging pathological phenotype of BSE (Wells *et al.*, 1992; Simmons *et al.*, 1996). The stability of the BSE strain has been further demonstrated by its

similar properties in inbred mice after primary transmissions from man (Bruce *et al.*, 1997; Hill *et al.*, 1997) and seven animal species, including the pig (Bruce *et al.*, 1994), that were naturally or experimentally infected with BSE. The findings of Bruce *et al.* (1994), Hunter *et al.* (1994) and other evidence summarized by Kimberlin (1994) indicate that the bovine PrP gene, and other bovine genes, are not significant variables in the occurrence or incubation period of BSE. Therefore, it is unlikely that variations in the donor species had major effects on the incubation period of BSE in pigs. There is less information on the potential effects of genetic variation in the recipients, but a previous study of six crossbred pigs, similar in their breeding to those used in the parenteral exposure experiment, revealed no sequence differences in the protein coding region of the PrP gene (Martin *et al.*, 1995). However, this does not exclude the possibility that variations in other regions of the PrP gene, or indeed other genes, may affect the incubation period of BSE in pigs.

Alternatively, the variable incubation periods may have been a consequence of multiple routes of inoculation that were used in this study, for two reasons. First, studies of the transmission of scrapie across the species barrier from sheep to mice showed that, with some isolates, the i.c. route failed to establish direct infection of the brain and

**Table 4.** Tissues inoculated into C57BLJ6 mice (assay end-point 700 days p.i.) or RIII mice (assay end-point 650 days p.i.) from the oral exposure study

Inocula were composed of single-tissue pools from multiple animals of exposed or control groups as indicated for the time-point ranges after exposure at which assays were conducted.

Tissue	Time after exposure when killed (weeks)	
	80–109*	350–371†
Brain (frontal cerebral cortex)	•	•
Brain (medulla)	•	•
Spinal cord (T <sub>10–11</sub> )	•	•
Semitendinosus M	•	•
Spleen	•	•
Thymus	•	•
Retropharyngeal LN	•	•
Mesenteric LN	•	•
Popliteal LN	•	•
Stomach	•	•
Distal ileum	•	•
Pancreas	•	•
Liver	•	•
Kidney	•	•

\*Five clinically normal exposed (380/91, 400/92, 406/92, 580/92 and 618/92) and five clinically normal control (148/92, 364/92, 373/92, 533/92 and 569/92) pigs; each tissue was assayed in C57BLJ6 mice as a pool from all exposed or control pigs.

†Three clinically normal exposed (1014/97, 1020/97 and 1029/97) and four clinically normal control (560/97, 1190/97, 1194/97 and 1303/97) pigs; each tissue was assayed in RIII mice as a pool from all exposed or control pigs.

that disease was a consequence of peripheral infection by inoculum that escaped from the site of injection (Kimberlin, 1993a). Secondly, compared to i.c. injection, greater volumes of inoculum can be injected by a combination of peripheral routes (i.v. and i.p.) to ensure a wide dissemination of

large amounts of infectivity to peripheral sites of agent replication in the lymphoreticular system (Millson *et al.*, 1979). It is possible that pig 58/90 had an exceptionally short incubation period because infection of the brain was established directly by the i.c. route, whereas this was not the case

**Table 5.** Summary of results of mouse (C57BLJ6) bioassay for pig tissues with confirmed infectivity in parenteral exposure study expressed as number of positive mice/number of mice surviving into the incubation period range\* (mean incubation in days  $\pm$  SEM)

Tissue	Pig no (weeks post-inoculation)			
	Initial clinical case	Pre-clinical cases		Pool of remaining four clinical cases
	58/90 (74)	36/91 (105)	38/91 (106)	341/91, 42/92, 66/92, 310/92 (139–163)
Brain (frontal cerebrum)	17/19† (594 $\pm$ 19.5)	16/19† (579 $\pm$ 34.5)	20/20† (544 $\pm$ 20.1)	17/20† (525 $\pm$ 22.7)
Spinal cord (C <sub>2–3</sub> )	–	17/20† (623 $\pm$ 21.6)	13/20† (585 $\pm$ 30.3)	–
Stomach	–	–	–	1/13† (708)
Jejunum	–	–	–	4/19† (603 $\pm$ 18.9)
Distal ileum	–	–	–	2/19† (637 $\pm$ 81.0)
Pancreas	–	–	–	1/14† (599)

\*No. of mice surviving beyond the time of death of the first positive mouse.

†No. of mice positive on immunohistochemical examination of assay mouse brains: brain (74) 19; brain (105) 18; brain (106) 20; brain (139–163) 17; spinal cord (105) 19; spinal cord (106) 17; stomach, 3; jejunum, 7; distal ileum, 7; pancreas, 2.

–, Bioassay not done.

in the other pigs whose incubation periods were, therefore, longer and fell into a narrower range. Another possibility is that i.c. injection was the direct cause of disease in all the affected pigs and that the variation in incubation period occurred because of differences in the precise site of injection, which is difficult to control without the use of stereotaxic procedures. Clear precedents for this are to be found in the results of studies of rodent models of scrapie (Gorde *et al.*, 1982; Kim *et al.*, 1987; Kimberlin *et al.*, 1987).

Infectivity was detected in the parenterally exposed pigs but not in the controls. Since all of the pigs were reared on a commercial ration containing MBM, this observation confirms that the occurrence of disease in the exposed pigs was due to the injection of the BSE agent. Infectivity was present in all the CNS tissues from exposed pigs that were tested (Table 5). However, not all of the assay mice injected with brain from clinically affected pigs developed the disease, and infectivity was detected in relatively few peripheral tissues. These findings suggest that a large species barrier to the transmission of BSE from pigs to mice compromised the sensitivity of the bioassays. Therefore, no conclusions can be drawn about the pathogenesis of BSE in pigs. The alimentary tissue infectivity in the affected pigs killed at 139–163 weeks p.i. (Table 5) could represent persistence of the inoculum (Millson *et al.*, 1979), replication of agent (Kimberlin, 1990), or centrifugal spread of agent from the CNS (Kimberlin *et al.*, 1983) in the late phase of the disease.

In contrast to the susceptibility of pigs to parenteral infection with BSE, exposure by feeding did not transmit the disease to pigs. This observation is in marked contrast to the now-considerable body of evidence that BSE has been transmitted, by natural or accidental means, via foodstuffs to several other animal species and to man (Bruce *et al.*, 1994; Kirkwood & Cunningham, 1994; Bruce *et al.*, 1997; Hill *et al.*, 1997). It has also been transmitted by feeding BSE-affected brain to mice (Barlow & Middleton, 1990) and mink (Robinson *et al.*, 1994) and by the oral dosing of sheep and goats (Foster *et al.*, 1993, Fraser & Foster, 1994) and cattle (Wells *et al.*, 1994, 1998). Transmission of BSE to lemurs (*Microcebus murinus*) after oral dosing with infected brain has been reported (Bons *et al.*, 1999). Thus, there is no doubt as to the susceptibility of several simple stomach species of mammals to BSE infection after oral exposure. The apparent exception of pigs is of considerable interest.

One possible explanation is that although the pig is susceptible to infection with the BSE agent, oral exposure was insufficient to establish infection. This is consistent with the findings from scrapie transmission studies within other species, which showed that the oral route of exposure is less efficient than parenteral routes (Kimberlin & Wilesmith, 1994). Other studies (see Kimberlin, 1996; Taylor *et al.*, 2001) make it likely that the effective exposure of pigs was further reduced by a species barrier to the oral transmission of BSE from cattle. The existence of a cattle–pig barrier can

be inferred from comparison of the present findings with the results of an oral titration in cattle of a pool of 60 BSE-affected brain stems. All the calves exposed to 100 g of brain developed clinical signs and histopathological lesions of BSE. At the time of writing, the amount of the brain pool required to cause BSE in 50% of the exposed cattle is estimated to be about 1 g or less (G. A. H. Wells & S. A. C. Hawkins, unpublished data). However, it should be noted that different pools of BSE brain were used in these two experiments. The infectivity titre of the pool fed to cattle was  $10^{3.5}$  mouse i.c./i.p. ID<sub>50</sub> units g<sup>-1</sup>, whereas the titre of the pool fed to the pigs was  $10^{2.4}$  mouse i.c./i.p. ID<sub>50</sub> units g<sup>-1</sup>, i.e. about 10 times lower. Thus, if the cattle–pig species barrier was zero, a dose of just over 10 g of brain should have caused disease in half of the exposed pigs. The fact that none of the pigs appeared to become infected after being fed an average of 400 g of brain on each of three successive occasions (a total of 1200 g) suggests the existence of a cattle–pig species barrier that reduced the effective oral exposure to BSE by as much as 100-fold, or even more.

A species barrier of such magnitude may be relevant to the fact that there have been no reports of a naturally occurring TSE in pigs in the UK, even though in the period that cattle were being exposed to contaminated MBM, pigs were also being exposed. Indeed, pigs continued to be exposed for several years after the introduction of the feed ban for ruminants in July 1988 (Anon., 1988). Moreover, the inclusion rates of MBM in commercial pig feeds were usually greater than in ruminant rations.

It is difficult to estimate the degree of BSE contamination of MBM. However, the epidemiological evidence suggests that the generally sporadic occurrence of cases throughout the BSE epidemic was the consequence of a relatively low-dose exposure (Wilesmith, 1991; Kimberlin & Wilesmith, 1994). Therefore, the risk of infection of any one animal at a given time was also low, even at the peak of the epidemic. The reason why a major epidemic occurred in the UK was because a large number of cattle received multiple potential exposures via contaminated MBM in concentrated feeds (Kimberlin, 1996). It was not possible to mimic the multiple low-dose exposures that pigs would have experienced naturally. Therefore, the design of the oral exposure study focused on three maximal exposures by replacing the MBM content of feed with brain material from cattle clinically affected with BSE. This exposure was greater than the exposure pigs could have received in the field, as is illustrated by the following calculations.

The BSE contamination of MBM used in feeds was dominated by the CNS from animals that had been infected as calves in dairy herds and were more than 2 years of age at slaughter (Kimberlin, 1996; Wells *et al.*, 1994, 1998). On average, the proportion of MBM derived from bovine material was 0.48 (MMC, 1985). Approximately 180 kg of waste material from each bovine was rendered to produce MBM (MMC, 1985) and less than 0.75 kg of this material was from the CNS. Therefore, the proportion of MBM

derived from bovine CNS was 0.2%. If 1% was the highest average proportion of all cattle going to slaughter that had been infected in dairy herds and were more than 2 years old (Kimberlin & Wilesmith, 1994; Wilesmith, 1991; Hoinville *et al.*, 1995), then the proportion of MBM derived from BSE-infected CNS would have been no more than 0.002% on average. In this study, the oral exposure to BSE was based on the consumption of 80 g MBM per day by commercially raised pigs (see Methods). In the field, CNS tissue would have contributed only about 1.6 mg of this amount. Therefore, replacing 80 g of MBM by whole brain increased the experimental exposure to CNS tissue to 50 000 times more than the calculated exposure in the field. Feeding such a large amount of BSE brain to pigs on just one of the three occasions probably represented an exposure well in excess of the cumulative lifetime exposure of pigs to BSE in the field (Kimberlin, 1996).

The evidence that exposure of commercial pigs to infected MBM did not result in cases of TSE (in contrast to the BSE epidemic in cattle) depends, *inter alia*, on the survival to maturity of a substantial population of animals. In 1983, there were 680 210 breeding sows in Great Britain (DAFS, 1983; MAFF, 1983), and in 1995 the number was 637 870 (MAFF, 1995; SOAEFD, 1995). Approximately 20% of these breeding pigs (~130 000), most of which were located in large commercial units, were kept until 4–5 years of age (MLC, 1999). If pigs were as susceptible as cattle to BSE by the dietary route, and with a similar median incubation period, then over 1000 cases of BSE in pigs should have occurred by now. Although there was no active surveillance for TSE in domestic pig populations in this country (or elsewhere), it is unlikely that many cases, had they occurred, would have escaped detection because the clinical signs of experimental BSE in the pig are distinctive. A description was published in February 1990 (Dawson *et al.*, 1990b) and the neurological signs may resemble those of some statutorily notifiable diseases in the pig.

Cases of TSE in pigs could have occurred undetected if incubation periods were much longer than for BSE in cattle. Unusually long incubation periods are often, though not invariably, found on transmission of TSE agents across a species barrier (Kimberlin *et al.*, 1989), and evidence for the existence of a cattle–pig barrier is discussed above. Nevertheless, the observations reported here on pigs orally exposed to exceptionally large amounts of infected brain and observed for 7 years thereafter support the view that the lower exposures encountered in the field were insufficient to cause infection and therefore, a naturally occurring TSE of pigs.

It is important to consider an alternative explanation of the outcome of the oral exposure experiment, because studies of several models of scrapie have shown that the disease may fail to develop, despite life-time persistence of infection in peripheral tissues (Dickinson *et al.*, 1975; Race & Chesebro, 1998), even at high titres (Bruce, 1985; Collis & Kimberlin, 1985). Therefore, it is possible that

infection of pigs occurred but did not produce clinical or pathological evidence of disease and the mouse bioassay was too insensitive to detect infectivity in any of the tissues.

This scenario can be examined by further consideration of the foodborne exposure of pigs to BSE in the field. Had primary BSE infection of pigs occurred from cattle, there would have been the potential for recycling and hence, amplification of a porcine-adapted BSE agent because of the inclusion in pig rations of MBM of porcine origin. This is directly analogous to the recycling that occurred in cattle and drove the bovine epidemic into an exponential phase (Kimberlin & Wilesmith, 1994; Wilesmith, 1991, 1998). Recycling in pigs would have occurred from the start of the BSE epidemic until April 1996, when legislation in Great Britain prohibited the feeding of mammalian MBM to all farmed animals (Anon., 1996). There would have been no species barrier to impede the transmission of any infection from pig to pig and had disease resulted, it might have been expected to occur with shorter incubation periods than primary foodborne transmission from cattle (Kimberlin, 1993b). The failure of recycling and amplification to produce clinical disease in pigs both before and currently, more than 6 years after the end of such exposure, tends to negate the hypothesis of inapparent BSE infection in pigs. Experimental investigation of this hypothesis would require subpassage of selected tissues, notably those of the alimentary tract, from the orally exposed pigs, employing the same species, or possibly transgenic mice expressing porcine PrP.

In conclusion, the present studies show that, although pigs are susceptible to BSE when injected by combined *i.c.*, *i.v.* and *i.p.* routes, there was no evidence of transmission after exposure by feeding three doses of BSE-infected brain in amounts equivalent to the maximum daily intake of MBM formerly used in commercial pig rations. The simplest explanation of this finding is that the effective exposure of pigs by the oral route was insufficient to establish infection. This explanation provides an understanding of why repeated primary exposures of commercial pigs to BSE, together with the considerable potential for pig-to-pig recycling of infection (until April 1996), has not resulted in natural cases of TSE in pigs. These observations are in contrast to the susceptibility of cattle to oral infection with gram quantities of BSE-affected brain and to the major feedborne epidemic in the UK.

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## REFERENCES

- Anon. (1988).** *The Bovine Spongiform Encephalopathy Order 1988*. Statutory Instrument 1988, No. 1039. London: HMSO.
- Anon. (1996).** *The Bovine Spongiform Encephalopathy (Amendment) Order 1996*. Statutory Instrument 1996, No. 962. London: HMSO.
- Baker, H. F., Ridley, R. M. & Wells, G. A. H. (1993).** Experimental transmission of BSE and scrapie to the common marmoset. *Vet Rec* **132**, 403–406.
- Barlow, R. M. & Middleton, D. (1990).** Dietary transmission of bovine spongiform encephalopathy to mice. *Vet Rec* **126**, 111–112.
- Bons, N., Mestre-Frances, N., Belli, P., Cathala, F., Gajdusek, C. & Brown, P. (1999).** Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. *Proc Natl Acad Sci U S A* **96**, 4046–4051.
- Bruce, M. E. (1985).** Agent replication dynamics in a long incubation period model of mouse scrapie. *J Gen Virol* **66**, 2517–2522.
- Bruce, M. E., Chree, A., McConnell, I., Foster, J., Pearson, G. & Fraser, H. (1994).** Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Phil Trans R Soc Lond Ser B Biol Sci* **343**, 405–411.
- Bruce, M. E., Will, R. G., Ironside, J. W. & 10 other authors (1997).** Transmissions to mice indicate that ‘new variant’ CJD is caused by the BSE agent. *Nature* **389**, 498–501.
- Collis, S. C. & Kimberlin, R. H. (1985).** Long term persistence of scrapie infection in mouse spleens in the absence of clinical disease. *FEMS Microbiol Lett* **29**, 111–114.
- DAFS (Department of Agriculture & Fisheries) (1983).** *Results of June 1983 Annual Agricultural and Horticultural Census, Scotland*. Edinburgh: DAFS.
- Dawson, M., Wells, G. A. H. & Parker, B. N. J. (1990a).** Preliminary evidence of the experimental transmissibility of bovine spongiform encephalopathy to cattle. *Vet Rec* **126**, 112–113.
- Dawson, M., Wells, G. A. H., Parker, B. N. J. & Scott, A. C. (1990b).** Primary parenteral transmission of bovine spongiform encephalopathy to the pig. *Vet Rec* **127**, 338–339.
- Dawson, M., Wells, G. A. H., Parker, B. N. J. & Scott, A. C. (1991).** Transmission studies of BSE in cattle, hamsters, pigs and domestic fowl. In *Subacute Spongiform Encephalopathies*, pp. 25–32. Edited by R. Bradley, M. Savelly & B. Marchant. Commission of the European Communities, Dordrecht: Kluwer Academic Publishers.
- Dawson, M., Wells, G. A. H., Parker, B. N. J., Francis, M. E., Scott, A. C., Hawkins, S. A. C., Martin, T. C., Simmons, M. M. & Austin, A. R. (1994).** Transmission studies of BSE in cattle, pigs and domestic fowl. In *Transmissible Spongiform Encephalopathies*, pp. 161–167. Edited by R. Bradley & B. Marchant. A Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels, 14–15 September 1993. Document VI/4131/94-EN. Brussels: European Commission, Agriculture.
- Dickinson, A. G., Meikle, M. H. & Fraser, H. (1968).** Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J Comp Pathol* **78**, 293–299.
- Dickinson, A. G., Fraser, H. & Outram, G. W. (1975).** Scrapie incubation time can exceed natural lifespan. *Nature* **256**, 732–733.
- Foster, J. D., Hope, J. & Fraser, H. (1993).** Transmission of bovine spongiform encephalopathy in sheep and goats. *Vet Rec* **133**, 339–341.
- Fraser, H. & Foster, J. (1994).** Transmission to mice, sheep and goats and bioassay of bovine tissues. In *Transmissible Spongiform Encephalopathies*, pp. 149–159. Edited by R. Bradley & B. Marchant. A Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels: 14–15 September 1993. Document VI/4131/94-EN. Brussels: European Commission, Agriculture.
- Fraser, H., McConnell, I., Wells, G. A. H. & Dawson, M. (1988).** Transmission of bovine spongiform encephalopathy to mice. *Vet Rec* **123**, 472.
- Fraser, H., Bruce, M. E., Chree, A., McConnell, I. & Wells, G. A. H. (1992).** Transmission of bovine spongiform encephalopathy and scrapie to mice. *J Gen Virol* **73**, 1891–1897.
- Gibbs, C. J., Jr, Gajdusek, D. C. & Amyx, H. (1979).** Strain variation in the viruses of Creutzfeldt–Jakob disease and kuru. In *Slow Transmissible Disease of the Nervous System*, vol. 2, pp. 87–110. Edited by S. B. Prusiner & W. J. Hadlow. New York: Academic Press.
- Gorde, J. M., Tamalet, J., Toga, M. & Bert, J. (1982).** Changes in the nigrostriatal system following microinjection of an unconventional agent. *Brain Res* **240**, 87–93.
- Hawkins, S. A. C., Ryder, S. J., Wells, G. A. H., Austin, A. R. & Dawson, M. (1998).** Studies of experimental transmissibility of BSE and scrapie to pigs. In *Proceedings of the 15th International Pig Veterinary Society Congress, Birmingham, July 5–9 1998*.
- Hill, A. F., Desbrusliers, M., Joiner, S., Sidle, K. C., Gowland, I., Collinge, J., Doey, L. J. & Lantos, P. L. (1997).** The same prion strain causes vCJD and BSE. *Nature* **389**, 448–451.
- Hoinville, L. J., Wilesmith, J. W. & Richards, M. S. (1995).** An investigation of risk factors for cases of bovine spongiform encephalopathy born after the introduction of the ‘feed ban’. *Vet Rec* **136**, 312–318.
- Hunter, N., Goldman, W., Smith, G. & Hope, J. (1994).** Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. *Vet Rec* **135**, 400–403.
- Kim, Y. S., Carp, R. I., Callahan, S. M. & Wisniewski, H. M. (1987).** Incubation periods and survival times for mice injected stereotaxically with three scrapie strains in different brain regions. *J Gen Virol* **68**, 695–702.
- Kimberlin, R. H. (1990).** Unconventional ‘slow’ viruses. In *Topley and Wilson’s Principles of Bacteriology, Virology and Immunity*, 8th edn, pp. 671–693. Edited by L. H. Collier & M. C. Timbury. London: Edward Arnold.
- Kimberlin, R. H. (1993a).** Modified pathogenesis of scrapie at first intracerebral passage from sheep to mice. In *Abstracts of the IXth International Congress of Virology*, P59-1, p. 318, Glasgow.
- Kimberlin, R. H. (1993b).** Bovine spongiform encephalopathy: an appraisal of the current epidemic in the United Kingdom. *Intervirology* **35**, 208–218.
- Kimberlin, R. H. (1994).** A scientific evaluation of research into bovine spongiform encephalopathy (BSE). In *Transmissible Spongiform Encephalopathies*, pp. 455–477. Edited by R. Bradley & B. Marchant. A Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels, 14–15 September 1993. Document VI/4131/94-EN. Brussels: European Commission, Agriculture.
- Kimberlin, R. H. (1996).** Bovine spongiform encephalopathy (BSE) and public health: some problems and solutions in assessing the risks. In *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases*, pp. 487–502. Edited by L. Court & B. Dodet. Paris: Elsevier.
- Kimberlin, R. H. & Wilesmith, J. W. (1994).** Bovine spongiform encephalopathy (BSE): epidemiology, low dose exposure and risks. In *Slow Infections of the Central Nervous System: the Legacy of Dr. Bjorn Sigurdsson*. Edited by J. Bjornsson, R. I. Carp, A. Love & H. M. Wisniewski. *Ann N Y Acad Sci* **724**, 210–220.
- Kimberlin, R. H., Field, H. J. & Walker, C. A. (1983).** Pathogenesis of mouse scrapie: evidence for spread of infection from central to peripheral nervous system. *J Gen Virol* **64**, 713–716.

- Kimberlin, R. H., Cole, S. & Walker, C. A. (1987).** Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral. *Microb Pathog* 2, 405–415.
- Kimberlin, R. H., Walker, C. A. & Fraser, H. (1989).** The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. *J Gen Virol* 70, 2017–2025.
- Kirkwood, J. K. & Cunningham, A. A. (1994).** Spongiform encephalopathy in captive wild animals in Britain: epidemiological observations. In *Transmissible Spongiform Encephalopathies*, pp. 29–47. Edited by R. Bradley & B. Marchant. A Consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities held in Brussels, 14–15 September 1993. Document VI/4131/94-EN. Brussels: European Commission, Agriculture.
- Lasmézas, C. I., Deslys, J.-P., Demalmay, R., Adjou, K. T., Lamoury, F., Dormont, D., Robain, O., Ironside, J. & Hauw, J.-J. (1996).** BSE transmission to macaques. *Nature* 381, 743–744.
- MAFF (Ministry of Agriculture, Fisheries & Food) (1983).** *Annual Agricultural and Horticultural Census, England and Wales*. London: MAFF.
- MAFF (Ministry of Agriculture, Fisheries & Food) (1995).** *Annual Agricultural and Horticultural Census, England and Wales*. York: MAFF.
- Martin, T., Hughes, S., Hughes, K. & Dawson, M. (1995).** Direct sequencing of PCR amplified pig PrP genes. *Biochim Biophys Acta* 1270, 211–214.
- Millson, G. C., Kimberlin, R. H., Manning, E. J. & Collis, S. C. (1979).** Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. *Vet Microbiol* 4, 89–99.
- MLC (Meat & Livestock Commission) (1999).** *MLC Pig Yearbook 1999*, p. 59. Milton Keynes: MLC.
- MMC (Monopolies & Mergers Commission) (1985).** *Animal Waste: a Report on the Supply of Animal Waste in Great Britain*, Appendix 2.1. London: HMSO.
- Race, R. & Chesebro, B. (1998).** Scrapie infectivity found in resistant species. *Nature* 392, 770.
- Robinson, M. M., Hadlow, W. J., Huff, T. P., Wells, G. A. H., Dawson, M., Marsh, R. F. & Gorham, J. R. (1994).** Experimental infection of mink with bovine spongiform encephalopathy. *J Gen Virol* 75, 2151–2155.
- Ryder, S. J., Hawkins, S. A. C., Dawson, M. & Wells, G. A. H. (2000).** The neuropathology of experimental bovine spongiform encephalopathy in the pig. *J Comp Pathol* 122, 131–143.
- Simmons, M. M., Harris, P., Jeffrey, M., Meek, S. C., Blamire, I. W. H. & Wells, G. A. H. (1996).** BSE in Great Britain: consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases. *Vet Rec* 138, 175–177.
- SOAEFD (Scottish Office, Agriculture, Environment & Fisheries Department) (1995).** *Results of June 1995 Annual Agricultural and Horticultural Census, Scotland*. Edinburgh: SOAEFD.
- Stack, M. J., Keyes, P. & Scott, A. C. (1996).** The diagnosis of bovine spongiform encephalopathy and scrapie by the detection of fibrils and the abnormal protein isoform. In: *Prion Diseases*, pp. 85–103. Edited by H. Baker & R. M. Ridley. Totowa, NJ: Humana Press.
- Taylor, D. M., Fernie, K., Steele, P. J. & Somerville, R. A. (2001).** Relative efficiency of transmitting bovine spongiform encephalopathy to RIII mice by the oral route. *Vet Rec* 148, 345–346.
- Wells, G. A. H., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., Dawson, M. & Bradley, R. (1987).** A novel progressive spongiform encephalopathy in cattle. *Vet Rec* 121, 419–420.
- Wells, G. A. H., Hawkins, S. A. C., Hadlow, W. J. & Spencer, Y. I. (1992).** The discovery of bovine spongiform encephalopathy and observations on the vacuolar changes. In *Prion Disease of Humans and Animals*, pp. 256–274. Edited by S. B. Prusiner, J. Collinge, J. Powell & B. Anderton. Chichester: Ellis Horwood.
- Wells, G. A. H., Dawson, M., Hawkins, S. A. C., Green, R. B., Francis, M. E., Simmons, M. M., Austin, A. R. & Horigan, M. W. (1994).** Infectivity in the ileum of cattle challenged orally with bovine spongiform encephalopathy. *Vet Rec* 135, 40–41.
- Wells, G. A. H., Dawson, M., Hawkins, S. A. C., Austin, A. R., Green, R. B., Dexter, I., Horigan, M. W. & Simmons, M. M. (1996).** Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy. In *Bovine Spongiform Encephalopathy. The BSE Dilemma*, pp. 28–44. Edited by C. J. Gibbs, Jr. New York: Springer-Verlag.
- Wells, G. A. H., Hawkins, S. A. C., Green, R. B., Austin, A. R., Dexter, I., Spencer, Y. I., Chaplin, M. J., Stack, M. J. & Dawson, M. (1998).** Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Vet Rec* 142, 103–106.
- Wells, G. A. H., Hawkins, S. A. C., Green, R. B., Spencer, Y. I., Dexter, I. & Dawson, M. (1999).** Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE). *Vet Rec* 144, 292–294.
- Wilesmith, J. W. (1991).** The epidemiology of bovine spongiform encephalopathy. *Semin Virol* 2, 239–245.
- Wilesmith, J. W. (1998).** *Manual on Bovine Spongiform Encephalopathy*, p. 51. FAO Animal Health Manual No. 2. Rome: Food and Agriculture Organization of the United Nations.
- Wilesmith, J. W., Wells, G. A. H., Cranwell, M. P. & Ryan, J. B. M. (1988).** Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec* 123, 638–644.