Further studies on the transmissibility of BSE to pigs

Introduction

Vacuolation, spongiosis, spongiform change or micro-cavitation are synonymous terms used to describe the light microscopic appearance of pathological optically empty spaces in histological sections of the central nervous system. Similar changes can be produced in histological preparations by autolysis and tissue processing artefacts (Wells and Wells, 1989). The occurrence of neuronal vacuolation, that is vacuoles within neuronal perikarya in certain neuroanatomical locations, as an incidental finding at post-mortem examination in the brains of domesticated animals, has been recorded previously and reviewed in relation to its potential to confound the histopathological diagnosis of transmissible spongiform encephalopathies (TSE) (Wells and McGill, 1992; Gavier-Widen et al., 2001). Although such occasional strictly localised neuronal vacuolation, especially in the dorsal nucleus of the vagus nerve, has been noted in pigs as an incidental finding in diagnostic submissions in the past (G. A. H. Wells, unpublished), a naturally occurring transmissible spongiform encephalopathy (TSE) has not been described in the pig. The observations of apparently incidental vacuolar changes also in neuropil (particularly that of the rostral colliculi) of the brains of exposed and control pigs in studies of the parenteral (project SE1816) and oral (project MO3004/SE1817) transmissibility of bovine spongiform encephalopathy (BSE) (Ryder et al., 2000; Wells et al., 2003) was therefore of interest in this context and because it does not seem to have been recorded previously in the pig or in other species.

The significance of such hitherto unreported vacuolation in the neuropil of the pig brain is unclear, although the most probable interpretation is that it represents an incidental background change of no pathological significance in the species. Nevertheless, it could be a confounding factor in the diagnostic histopathological examination of pig brains, particularly so if such examinations were being conducted in the course of any surveillance of at risk pig populations for evidence of a TSE.

One part of study M03010 aimed to obtain information on the geographical and temporal occurrence of vacuolation in the grey matter of the brains of pigs and thereby help to clarify the potential significance of such changes. It was directed primarily to the vacuolar changes previously observed in the rostral colliculus. The studies examined archived diagnostic material, accessed opportunistically according to availability and prospective material, sourced from healthy slaughtered animals.
In the second part of this study, samples of rostral colliculus from porcine brains featuring vacuolar changes were used to inoculate pigs parenterally to assess the biological significance of such changes. These brains were from pigs born

i) in Great Britain during a period when they would have been fed a commercial ration which may have contained meat and bone meal (MBM).

ii) in Great Britain during a period after the exclusion of MBM from commercial rations.

iii) in New Zealand a country free of animal TSE (negative control)

This transmission study would also help to elucidate whether an endemic TSE like agent or the agent responsible for the BSE epidemic may be responsible for the observed vacuolation in the brain.

In addition, for the purpose of tissue production and to provide a positive control group, pigs were challenged with the brain stem homogenate from cattle affected by BSE.

Further subsidiary components of work funded under this project aimed

i) to test the brains of pigs fed BSE affected brain (from previous project SE1817/MO3004), for disease specific PrP using the antiserum 1B3 (to complement studies of PrP detection in the brains of parenterally inoculated pigs in SE1816 using this antiserum)

ii) to test the efficacy of a range of PrP antibodies for detection of disease specific PrP in the pig brain

iii) to examine the vacuolated rostral colliculus in pigs for evidence of a glial response that would indicate a pathological process at this location.

Materials and Methods

Histological study of pig brains

Rationale for the selection of material

The age range at which the vacuolar change was detected in the experimental study of the transmissibility of BSE to pigs was 49-265 weeks (approximately 1-5 years). Because of interest in the remote possibility of an association of such changes with a transmissible spongiform encephalopathy in the latter situation and the long incubation periods of such diseases, this study was directed predominantly toward examination of pigs over one year old, but material from some younger pigs was also examined.

Geographical sources of material
Haematoxylin and eosin (HE) stained sections of pig brains were examined from archives of veterinary pathology resources in Great Britain, Germany and New Zealand. Prospective studies were also conducted on the brains from samples of clinically normal pigs from Great Britain, Germany and New Zealand.

Archived material
Routine HE stained sections prepared from diagnostic submissions for histopathological examinations in which the rostral colliculus was represented were examined by light microscopy. As far as was possible adult animals were selected, but where these were not available from a source suitable material was examined from younger pigs.

- Germany
  - Paraffin wax-embedded blocks from the brains from twenty six pigs were received.
- New Zealand
  - Paraffin wax-embedded blocks from the brains from twenty six pigs were received.
- Great Britain
  - The brains from twenty two pigs held within the VLA archive were examined.

Prospective studies
- New Zealand:
  - Twenty whole brains fixed in 10% formal saline were received from healthy pigs slaughtered (requested > 1 year old, but age not specified) in 1997.
- Germany:
  - Ten whole brains fixed in 10% formal saline were received from healthy sows slaughtered in 1997.
- Great Britain:
  - Ten clinically normal adult pigs, designated for culling, were sourced from commercial breeding units. The pigs were killed, whole brains removed and fixed in 10% formal saline.

Coronal blocks of tissue representing all major brain regions were cut and processed routinely to 5µm HE sections for light microscopic examination.

Transmission study

Preparation of inocula pools and inoculation groups
Piglets were allocated at random to the challenged groups:
1. Pre-1996 and Post-1996 test groups

Rostral colliculi of 21 normal healthy culled sows sourced in Great Britain, ten born prior to 1996 and eleven born after 1996, were dissected aseptically and one half retained for histological examination and the remainder frozen. Selection of these source groups was based on the premise that the pigs born prior to 1996 were fed a commercial ration, which may have contained MBM whereas pigs born after 1996 were fed a commercial ration in which the inclusion of MBM was prohibited. The frozen tissues from the respective time periods were pooled to form two inocula.

Ten 2-3 week-old commercial large white pigs were inoculated parenterally, each with one of the pooled inocula (four female, six barrows in each group) as described in an earlier study (Wells et al., 2003). Briefly, brain material for each group was pooled, prepared as 10% homogenate in sterile saline solution, and administered intracerebrally (0.5 ml), intravenously (1.5 ml) and intraperitoneally (8 ml).

2. New Zealand (NZ) group

Brains of 13 culled sows from NZ, a country free from animal TSE, were sampled aseptically and the rostral colliculi pooled for inoculation. The brains of an additional five pigs from the same source were fixed in 10 per cent formol saline for subsequent histopathological examination of the rostral colliculus. Ten 2–3 week-old pigs (seven female, three barrows) were inoculated similarly to the test groups with the pooled rostral colliculus inoculum.

Due to the late availability of NZ-derived brains, pigs in this control group were inoculated approximately four months later than the other groups.

3. Positive control group (BSE group)

A pool of brainstems collected aseptically from two cattle affected by BSE and culled in 1999 was used for parenteral inoculation of a further ten piglets. The infectious titre of this brain pool was not determined.

One male piglet died on the day of inoculation; its replacement was an unchallenged female piglet, which was mixed with the BSE-challenged animals.

Husbandry
Pigs of each group were housed separately throughout the study and had no nose-to-nose contact. Separate entrances for each group of pigs, together with dedicated protective clothing and animal husbandry equipment for each group was also maintained throughout the study. Initially, both challenged groups were housed in one building and shared the same air space whilst the NZ and BSE groups were housed in separate buildings attended by different animal husbandry staff. The pigs were kept in pens, each designed to hold two pigs, but some pigs were kept on their own to avoid aggression. Between 98 and 114 weeks post inoculation (wpi), pigs were moved to a single building, which had four separate pens with separate entrances for each challenged group but a common air space; all pigs were cared for by the same animal technicians. Pigs were reared and maintained throughout the study period on a pig ration free from animal protein, which was routinely monitored.

Clinical monitoring

Pigs were monitored daily by animal husbandry staff. More detailed clinical monitoring commenced at between 33 and 52 weeks post inoculation (wpi). Clinical assessments were usually conducted monthly and comprised assessments of behaviour (response to approach by a human), sensation/vision (response to pricking of the neck with artery forceps and evaluation of the response to a threatening gesture towards the eye [menace response]) as well as locomotion. Clinical signs recorded in detail were over-reactivity to external stimuli or to approaching, such as flinching, grunting, squealing and/ or moving away. Apprehension was defined as grunting, squealing or running away with vocalising when approached by the observing person. A prion disease was suspected when the following signs were displayed: a combination of apprehension and gait abnormalities (stiff or ataxic gait) or a combination of over-reactivity to external stimuli (menace response testing and response to neck prick) and gait abnormalities in the absence of other abnormalities that could have explained these signs. This was based on the description of the signs of BSE in a previous study (Wells et al., 2003; Wells et al., 2006) and followed similar criteria as for the diagnosis of BSE in cattle where behavioural, sensory and locomotor changes are associated with BSE (Braun et al., 1998; Konold et al., 2004).

The animals were monitored for five years after inoculation and then culled unless they displayed clinical signs suggestive of a TSE or any other intercurrent disease that required an earlier cull. The clinical assessments were initially carried out “blind” without knowledge of the inoculation status, when they were housed in separate buildings. Knowledge of the positive and negative control group was unavoidable after they moved to the new accommodation although the identity of the
unchallenged pig mixed with the positive controls remained unknown. The frequency of expressed clinical signs in pigs of each group prior to cull was compared by Fisher’s exact test.

**Postmortem examination**

The brains of all pigs were examined by routine histopathology for the presence of spongiform encephalopathy and by immunohistochemistry (IHC) as well as Western immunoblot (WB) for the presence of disease-specific prion protein (PrP\(^d\)) or its proteinase-resistant form, PrP\(^\text{res}\). Brain sections subject to a neuropathological examination were the brainstem at the level of the obex and the rostral midbrain. In three pigs with neurological signs, additional sections comprising the cerebellum, the frontal and the parietal cerebrum were processed. Initially, the monoclonal antibody (mAb) used for IHC was mAb L42 (R-Biopharm AG, Darmstadt, Germany), which has been used successfully for detection of PrP\(^d\) in sheep and cattle (Hardt et al., 2000). This antibody had shown similar disease-specific immunolabelling in BSE affected pig brain as the polyclonal mouse antiserum 1B3 used in an earlier experiment (Ryder et al., 2000) but is consistently associated with relatively high levels of non-specific background immunolabelling (J Spiropoulos and GAH Wells, unpublished observation). Subsequently, mAb 2G11, (Institut Pourquier, Montpellier, France), which had been used successfully for the diagnosis of atypical scrapie of sheep (Simmons et al., 2007), was evaluated and gave high levels of specific immunolabelling and negligible non-specific labelling in porcine BSE-affected brains and is now considered the antibody of choice for the identification of PrP\(^d\) in porcine samples (J Spiropoulos and YI Spencer, unpublished observation). This antibody was used in addition to L42 on brain sections of any pig testing positive by histopathological, WB or initial IHC examinations, on brain sections of an unchallenged control pig, and on brain sections of the three pigs that were subject to further neuropathological examination (see above).

For the WB, the VLA Hybrid technique with mAB 6H4 and P4 was used on a fresh sample of the caudal medulla (Stack, 2004).

**Additional studies**

Earlier work as part of project SE1816 demonstrated disease-specific PrP accumulation in the brains of pigs inoculated parenterally with BSE brain homogenate (Ryder et al., 2000). This was achieved immunohistochemically using a rabbit anti PrP antiserum, 1B3. At the time of the study this was the
only reagent available that was able to identify PrP in the brains of pigs. In order to examine similarly the brains of pigs in a previous study which were fed with BSE affected brain (SE1817), brain sections from all orally challenged animals and the control group killed after seven years were immunolabelled using the protocol developed for affected animals from SE1816, utilising antiserum 1B3.

The supply of 1B3 antiserum was subsequently exhausted, and future use of IHC on suspected TSE affected pig material required the identification of alternative reagents. New anti PrP antibodies were being generated in laboratories around the world, and one of the objectives in this study was to assess candidate antibodies for use in immunolabelling PrP in pig brain. Of the antibodies that became available several had shown a wide species range of reactivity and five were tested on pig material derived from the successful challenge of pigs with BSE (SE1816). [NB this part of the study was conducted before the mAb 2G11 became available – see above]

The antisera tested were:

- **Rb486.** Polyclonal rabbit antiserum, generated at VLA to a synthetic peptide equivalent to bovine PrP residues 230-244 and used as principal reagent for TSE diagnosis in cattle and sheep. It has also been used successfully for the identification of PrP in the brains of mice inoculated with BSE agent (Wells et al., 2003).
- **F89 and F99.** Mouse monoclonal antibodies (mAb) produced by Dr Katherine O’Rourke, USDA. Both of these reagents produce good results in sheep, but show high background in cattle. This mAb also immunolabels PrP in the brains of cats with FSE.
- **R145.** A rat mAb generated at VLA Weybridge which proved extremely effective in labelling PrP in cattle and sheep and discriminating between disease-specific and background reactivity.
- **L42.** A mAb produced by Dr M Groschup, FRCVDA, Tübingen, Germany. This was an effective reagent for use in both cattle and sheep and the likely candidate for a common EU immuno-diagnostic protocol.

To investigate the potential pathological significance of vacuolation of the rostral colliculi in normal pigs, anti GFAP staining was carried on the midbrain sections of selected control animals from project SE1817. These comprised four two-year old pigs and four seven-year old animals, all of which showed neuropil vacuolation of the rostral colliculi. BSE affected pig midbrain was included
as a positive control.

Results

Histological study

The frequency of occurrence of vacuolar changes in the neuropil of the rostral colliculus in the various samples acquired is given in Table 1. Although the vacuolation was invariable relatively sparse, recorded only rarely as moderate, when sampling allowed examination of some sub-adult or younger animals there was a general trend of greater intensity of vacuolation at the site in adult animals. It was recorded in a high proportion of pigs from all geographical locations and in those submitted with systemic illness (diagnostic submissions) and those healthy at slaughter. The archived samples were from animals killed over almost a 50 year period (1952-1991) and prospectively acquired material was from animals killed in the period 1996-1997. While breed was not recorded in many cases, the available data and commercial sources indicate that most were either purebreeds such as Landrace or Large White, or commercial hybrids.

Transmission study

Histopathological examination of the brains of test group animals and UK donor pigs revealed neuropil vacuolation of the rostral colliculus, which was also present in the brains of pigs sourced from NZ. This vacuolation resembled closely that described in healthy pigs of other studies (Ryder et al., 2000; Jahns et al., 2006). Vacuolar changes were also observed in brainstem sections at the level of the obex of all inoculated pigs, which was also reported in these studies.

Table 2 lists individual details and pathological status of the animal used in the study. In the brain sections examined, disease-specific immunolabelling was present in only two pigs of the BSE group. Figures 1d-i represent the rostral midbrain from a BSE positive pig (PR443) with disease-specific immunolabelling using both mAb L42 and 2G11; Figures 1a-c are examples of pigs in this study, which did not present with disease-specific immunolabelling (BSE negative). See figure legends for more details. PrP
d was detectable by WB in the same two pigs of the BSE group in which disease-specific immunolabelling was found on IHC, but was not detectable in the other pigs (see Figure 2).
The frequency of assessed clinical signs within each group prior to cull is displayed in Table 3. Statistical significant differences were only seen between the BSE-inoculated pigs and the other groups ($P<0.05$).

Based on the presence of apprehension or over-reactivity to external stimuli and gait abnormalities, which were associated with a prion disease, eight pigs were classified as TSE suspects prior to cull. These pigs belonged to the BSE group and included the two pathologically confirmed BSE cases.

**Pathologically confirmed BSE cases**

**PR463**
At 143 wpi, this pig developed over-reactivity to tactile stimuli, which were consistent on subsequent examinations. It also developed a stiff gait at that time and was finally found recumbent with difficulty rising, which led to its cull at 148 wpi.

**PR443**
This pig developed over-reactivity to visual stimuli at 157 wpi, which continued to be observed on subsequent assessments, followed by stiffness of the gait. It eventually developed ataxia with hind limb weakness resulting in difficulty getting up; a whole body tremor was also present prior to cull at 175 wpi.

**BSE-inoculated pigs without pathological confirmation and comparison with pigs from other groups**

Generally, differences in the behaviour of pigs in the BSE group compared to the other groups were observed after transport to new accommodation at 115 wpi; they appeared to be more nervous and over-reactive.

This was most marked in two pigs, PR442 and PR444, which became very apprehensive and over-reactive, characterised by loud squealing and running away in panic whenever approached or touched. This behaviour had not been seen in their previous accommodation. However, these two pigs appeared more inquisitive on occasions, when they would approach an observer with repeated short grunts or would follow an observer with repeated grunting and muzzling although any attempt to touch the animal would result in immediate withdrawal with squealing. Eventually, these characteristic repeated short grunts were also elicited by merely entering the pen, and other pigs would join in. Similar vocalising behaviour was also expressed by the unchallenged control pig kept with the BSE-challenged pigs – and to a much lesser degree – in some animals in the other three groups, although this did not coincide with the panic expressed when the animals were approached
themselves.

Behaviour, such as squealing, grunting, ear flapping or running away when approached or when the response to external stimuli was tested, was more frequently and consistently displayed in the BSE-challenged animals compared to pigs in the other groups, with the exception of one animal, PR462, which displayed this behaviour only inconsistently.

Ataxia was observed in four BSE-challenged, pathologically unconfirmed pigs from between 218 and 281 wpi, including PR462 and PR442. The latter was also very over-reactive and apprehensive. Although the other markedly over-reactive animal, PR444, did not display ataxia, it had developed a stiff gait 11 weeks prior to cull.

The combination of over-reactivity and ataxic or stiff gait were exhibited by eight BSE-challenged animals, including the two pathologically confirmed cases (see Table 3, animals PR440, PR441, PR442, PR443, PR444, PR445, PR462 and PR463).

Five animals of the BSE-challenged group displayed the combination of apprehension and an ataxic or stiff gait (PR440 PR441 PR442 PR444 and PR445). It was also displayed in one animal from the other Post-1996 group, PR488. This pig presented with ataxia from 229 wpi, which coincided with the appearance of and treatment for an abscess on the dorsal neck. This pig continued to be incoordinated when culled 26 weeks later due to a foot abscess and became apprehensive but was not over-reactive to external stimuli. As the display of apprehension coincided with the treatment (the pig was not apprehensive prior to treatment and disease), this sign was not considered to be associated with a prion disease. Multiple abscesses were also found in the abdomen.

Histopathological examination of a section of the cervical and thoracolumbar spinal cord did not reveal any conclusive changes that could have accounted for the observed gait abnormality.

All five animals of the BSE-challenged group that displayed apprehension and an ataxic or stiff gait were also over-reactive to external stimuli.

PR460 was the only BSE-challenged pig not to display the combination of signs considered to be suggestive of a prion disease. This pig displayed over-reactivity to external stimuli prior to cull, which included both over-reactivity to the menace response testing and over-reactivity to neck pricking. Over-reactivity to both of these tests were also displayed by PR440, PR442, PR444 (BSE-challenged group) and PR472 (Pre-1996 group).

PR593 (NZ group) developed an abscess in the spinal cord and became recumbent with paresis of the hind limbs but was not ataxic prior to this event.
Additional clinical findings in pigs

Twenty-two pigs presented with an ear tremor, which was either noticed on the first clinical assessment or developed over time. This tremor occurred in pigs of all groups (BSE group: 3, NZ group: 3, Pre-1996 group: 8, post-1990 group: 8) and was most pronounced when pigs were stressed or excited whereas it was not evident in a quietly resting or sleeping pig.

Additional neuropathological examinations

The brains of three pigs from the BSE group that were markedly apprehensive and displayed an ataxic or stiff gait (PR440, PR442, PR444) were examined neuropathologically in more detail to determine the cause of the changes since BSE was not confirmed by postmortem tests. Histopathologically, there was mild to moderate neuropil vacuolation of the rostral colliculus in all cases. Additional findings were localised mild white matter vacuolation within the roof nuclei of the cerebellum (PR442) and in the thalamus (PR440, PR444), single vessels showing perivascular mononuclear cell infiltration (PR440, PR442, PR444) and focal mineralisation in meninges (PR444). These changes were not considered to be clinically significant due to their limited extent and severity.

PrP\textsuperscript{D} immunolabelling using MAB 2G11 revealed variable mild fine filamentous labelling at some brain locations (cingulate gyrus, PR440; ventro-lateral frontal cortex, PR444; hippocampus, PR442). Faint granular cytoplasmic labelling was visible occasionally in neurons (lateral tegmentum of midbrain and hippocampus in PR442; hypothalamus, hippocampus, medial geniculate nucleus, substantia nigra, oculomotor nucleus and central grey matter at level of midbrain, reticular formation in rostral medulla in PR444). Such labelling has been observed previously in sheep (Ryder et al., 2001) and cattle (GAH Wells, unpublished observation) unaffected by TSE.

Additional studies

Immunohistochemical examination of the brains of pigs exposed orally to BSE agent (SE1817) for disease-specific PrP

Non-specific immunolabelling was observed and comprised peri-neuronal labelling of neurons in the dorsal trapezoid nucleus in 3/5 control and 4/9 challenged animals. Peri-neuronal labelling was also seen in the vestibular nuclei of one pig, challenged and killed after two years. There were no significant difference between control and challenged groups, and no evidence of disease-specific
PrP accumulation. This supported the histopathological findings from the study that there is no evidence of transmission of BSE to the pig by the oral route.

**Testing of PrP antibodies**

Of these reagents tested only L42 produced disease-specific PrP labelling in porcine CNS. This took the form of particulate neuropil labelling, the same as that seen in other species and in the parenterally inoculated pig material using 1B3. The distribution of labelling with this mAb was also closely similar to that with antiserum1B3. Of the other antibodies tested F89 gave high background non-specific labelling. L42 was therefore used to investigate the presence of disease-specific PrP in this study.

**Examination of vacuolated rostral colliculus in pigs by GFAP staining**

In none of the control pigs examined was there any evidence of astrocytic changes, whereas in the BSE affected animal hypertrophy of astrocytes was detected.

**Discussion**

**Histological study**

It appears, given the frequency of neuropil vacuolation in the rostral colliculus in archived diagnostic submissions and in healthy pigs from commercial sources in diverse geographical locations that it has undoubtedly been observed by veterinary pathologists in the past, but has been dismissed as an insignificant feature and not recorded. It is likely that the relatively mild nature of this change (in no instance was it recorded as severe and in many the change is represented by very few vacuoles) has contributed to this perspective. It appears that the vacuoles may increase with age. The occurrence of the change in retrospective material sourced from diverse geographical locations would suggest that it has been a feature of commercial pigs for many years. Clearly, such vacuolation has not been associated with any observed clinical sign.

The absence of a demonstrable astrocytic response in the vacuolated rostral colliculus of normal experimental control pigs supports the conclusion that the vacuolation does not evoke a demonstrable glial response and is therefore of doubtful pathological significance. The result also argues against the phenomenon being a result of incipient TSE infection in this species.
Transmission study

Only two of nine pigs inoculated with BSE-affected brain developed a prion disease that was confirmed by postmortem tests. The poor attack rate may be explained by the species barrier between cattle (donor of inoculum) and pigs (recipients of inoculum), or it may be that a higher titre of inoculum is required to achieve a greater attack rate. The effect of decreasing titres of a BSE inoculum on incubation period and attack rate has been well documented in wild-type mice (Green et al., 2005). The inoculum was not titrated in mice to determine the infectious titre but both BSE-positive pigs were culled with clinical signs at 148 and 175 wpi compared to a range of 74 to 163 wpi in a previous study (Wells et al., 2003). Although, in this previous study, the pooled inoculum (made from four BSE-affected cattle brainstems [BSE1–4]) was also not titrated, transmission studies in mice indicate that the tissue that contributed to the pool had a titre of approximately $10^5$ intracerebral (i.c.) ID$_{50}$ mouse infectious units per g (Bruce et al., 1994; Wells et al., 2003). Furthermore, the transmission rate in the pigs was high (eight of eight pigs surviving beyond 50 wpi presented with a prion disease clinically and/or pathologically). While the cull times in the present study might therefore suggest that the infectious titre of the inoculum was probably slightly lower than for the previous study, this does not provide a satisfactory explanation for the low attack rate given the optimal parenteral routes of inoculation used in both studies.

There was no significant difference in the (mildly expressed) clinical signs between pigs inoculated with NZ-derived brain and the pre-1996, post-1996 test groups, whereas the clinical signs in the BSE-inoculated pigs (positive control group) were significantly different and included signs of a neurological disease. Histopathological and immunohistochemical examination of brains from the rostral colliculus-inoculated test groups revealed no prion disease-specific vacuolation or immunolabelling, and PrP$^{res}$ was also not detected by Western immunoblot. The findings suggest that vacuolation in this species is not associated with a prion disease and is biologically or clinically irrelevant.

Eight of the nine pigs in the positive control group were considered to be affected by a neurological disease at the time of termination of the study (approximately 290 wpi). BSE was suspected in these pigs because the clinical signs were similar to those seen in the pigs culled at 148 and 175 wpi and to those reported in previous studies (Dawson et al., 1990; Wells et al., 2003) or documented on video tape of the first case of experimental BSE in the pig (MAFF, 1990). For example, pigs with BSE in a previous study became easily frightened or persistently approached attendants with continual
vigorous vocalisations (Wells et al., 2006), which was similarly observed in the majority of the BSE group in this study. The presence of behavioural or sensory changes in combination with an abnormal gait is frequently found in BSE-affected cattle, and this finding in the BSE-challenged pigs in the absence of any other disease that could have explained these signs was suggestive of a prion disease.

The clinical protocol used for this study differed slightly from the protocol used in previous pig studies (no previous testing of the response to external stimuli) because it was adapted from the assessment of cattle for signs of BSE, grouped into behavioural, sensory and gait changes (Konold et al., 2004). The various vocalisations of pigs were particularly assessed since vocal responses have been frequently used to study behaviour and stress in pigs (Marchant et al., 2001; Düpjan et al., 2008). Squealing is associated with increased stress or pain in pigs (White et al., 1995; Weary et al., 1998; Houpt, 1998) and was in this study interpreted as increased over-reactivity (when in response to external stimuli) or apprehension (when in response to an observer’s approach). Short rapidly-repeated grunts as produced by pigs in the BSE group – and to a lesser degree by pigs in the other groups – appear to have a greeting or threat function (Marchant et al., 2001). Aggressiveness was however not observed in these pigs; in fact, the pigs would run away when approached. These vocalisations occurred later in the incubation period and - in combination with the squeals and grunts after stimulation of the animals - were interpreted as behavioural changes.

The combination of behavioural, sensory and gait changes, seen in five of these pigs, was suggestive of a brain disease (Lorenz and Kornegay, 2004); behavioural or sensory changes observed in the other pigs of this group without clear neurogenic gait deficits may have been caused by a disease not affecting the brain. To investigate the cause of the observed neurological signs the brains of three pigs with the most severe clinical signs were subject to a more detailed neuropathological examination. The histopathological changes in the brains of these pigs were either considered a “normal” feature of adult and sub-adult pigs (Ryder et al., 2000; Wells et al., 2003; Jahns et al., 2006) or regarded as changes of no clinical significance due to their limited extent and severity, which would be anticipated in normal pigs. Faint granular cytoplasmic immunolabelling had also been found in pig brains in project M03005 (oral exposure of pigs to scrapie ) in which there was no evidence of transmission. We were unable to determine the cause for the observed clinical signs although the presence of clinical signs in BSE-infected pigs culled at termination of the study, which were not observed similarly in the other groups, raises the possibility that these animals may have developed a non- or not yet fatal prion disease that remained undetected by the current statutory diagnostic tests.
A transmission study of bovine BSE in transgenic mice expressing porcine prion protein (poTg mice) failed to induce disease and detectable PrP\(^d\) accumulation in mice on first passage but subsequent passage of brain homogenates from these mice in poTg mice produced a prion disease with clinical signs (Castilla et al., 2004). The authors concluded that there was a substantial species barrier between cattle and pigs but exposure to bovine prions may lead to subclinical infection. This is in contrast to our findings where pigs showed neurological signs suggestive of a TSE without pathological evidence of disease. Further transmission of brains from clinically affected pigs to pigs or poTg mice to overcome the species barrier would be necessary to determine whether these pigs were indeed affected by a prion disease. Clinical signs of a prion disease have been observed in mice infected with prions in the absence of detectable prion protein (Lasmézas et al., 1997; Barron et al., 2007).

Congenital tremor has been well described in pigs and may be hereditary (e.g. in the Landrace pig) or caused by infections (Harding et al., 1973; Dewey, 2006). It usually involves the legs, the body and the head and may disappear with increasing age. The tremor seen in the pigs of this study were only confined to the head and affected predominantly the ears, with the exception of the confirmed BSE case PR443, which exhibited a body tremor prior to cull. Tremor in the shoulder regions, flanks and of the ears has also been observed in pigs affected by BSE (Wells et al., 2006). In our study, not all pigs in one litter were affected but occurrence in pigs of all groups is not suggestive of an association with a spongiform encephalopathy. That the tremor, at least in some cases, appeared to develop over time, is suggestive of an acquired rather than a congenital disorder. Intracranial challenge may occasionally cause neurological abnormalities, such as medial strabismus and exophthalmos in cattle inoculated intracerebrally (T. Konold, unpublished observation). However, no significant abnormalities were detected by histopathological examination of caudal brainstem and the rostral midbrain (or in other brain regions of one pig [PR440]) of the pigs affected by tremor.

**Conclusion**

The findings suggest that vacuolation in the brains of pigs is common without causing evident clinical signs and does not represent a transmissible spongiform encephalopathy. The presence of neurological signs in pigs challenged with BSE in the absence of detectable disease-associated prion protein or other visible pathological changes raises the possibility that the BSE agent may cause a chronic disease that remains undetected by current prion disease phenotypic definitions and
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Table 1. Frequency of occurrence of vacuolar changes in the neuropil of the rostral colliculus in diagnostic submission (archive cases) and slaughtered pigs (prospective samples) according to geographical source and period of sampling

<table>
<thead>
<tr>
<th>Source</th>
<th>Approximate period of survey sample accession</th>
<th>Age range</th>
<th>Frequency of occurrence of vacuolar changes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Archive cases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand¹</td>
<td>1970-1991</td>
<td>3month – Adult</td>
<td>5/7</td>
</tr>
<tr>
<td>New Zealand²</td>
<td>1952-1985</td>
<td>Neonate – “Porker”</td>
<td>9/16</td>
</tr>
<tr>
<td>Germany³</td>
<td>1996-1997</td>
<td>5 month – 3.5 years</td>
<td>7/15</td>
</tr>
<tr>
<td>Great Britain⁴</td>
<td>1959-1990</td>
<td>Adult</td>
<td>22/22</td>
</tr>
<tr>
<td><strong>Prospective Samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand⁵</td>
<td>1997</td>
<td>&gt;1 year</td>
<td>15/20</td>
</tr>
<tr>
<td>Germany⁶</td>
<td>1997</td>
<td>&gt;2 years</td>
<td>8/10</td>
</tr>
<tr>
<td>Great Britain⁷</td>
<td>1996-1997</td>
<td>Adult</td>
<td>10/10</td>
</tr>
</tbody>
</table>

¹ Archived case material in which blocks of rostral colliculus were available, provided by Pathology Department, Massey University, Palmerston North, New Zealand.

² Archived case material in which blocks of rostral colliculus were available, provided by Batchelar Animal Health Laboratory, Palmerston North, New Zealand.

³ Archived case material in which blocks of rostral colliculus were available provided by Institute of Pathology, Hanover, Germany.

⁴ Archived case material in which blocks of rostral colliculus were available from Pathology Department, VLA Weybridge.

⁵ Healthy slaughtered pigs provided by MAF Quality Management, New Zealand.

⁶ Healthy slaughtered German Landrace hybrid provided by University of Giessen, Germany.

⁷ Slaughtered adult pigs (cull sows) sourced from commercial breeders in GB.
Table 2. Individual details and pathological status of the pigs used in the transmission study

<table>
<thead>
<tr>
<th>Animal identification</th>
<th>Inoculum</th>
<th>Sex</th>
<th>wpi</th>
<th>Experimental outcome</th>
<th>Postmortem result (HP/IHC/WB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR459</td>
<td>None</td>
<td>F</td>
<td>N/A</td>
<td>Intercurrent death (arthritis)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR471</td>
<td>Pre-1996</td>
<td>M</td>
<td>250</td>
<td>Intercurrent death (foot abscess)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR486</td>
<td>Pre-1996</td>
<td>F</td>
<td>265</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR484</td>
<td>Pre-1996</td>
<td>M</td>
<td>277</td>
<td>Intercurrent death (arthritis &amp; spondylosis)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR470</td>
<td>Pre-1996</td>
<td>M</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR472</td>
<td>Pre-1996</td>
<td>M</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR475</td>
<td>Pre-1996</td>
<td>F</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR478</td>
<td>Pre-1996</td>
<td>F</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR482</td>
<td>Pre-1996</td>
<td>F</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR467</td>
<td>Pre-1996</td>
<td>M</td>
<td>297</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR469</td>
<td>Pre-1996</td>
<td>M</td>
<td>297</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR477</td>
<td>Post-1996</td>
<td>F</td>
<td>209</td>
<td>Intercurrent death (cause undetermined)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR480</td>
<td>Post-1996</td>
<td>M</td>
<td>250</td>
<td>Intercurrent death (tumour)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR488</td>
<td>Post-1996</td>
<td>M</td>
<td>255</td>
<td>Intercurrent death (foot abscess)</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR468</td>
<td>Post-1996</td>
<td>M</td>
<td>295</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR479</td>
<td>Post-1996</td>
<td>M</td>
<td>296</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR481</td>
<td>Post-1996</td>
<td>M</td>
<td>296</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR483</td>
<td>Post-1996</td>
<td>F</td>
<td>296</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR487</td>
<td>Post-1996</td>
<td>F</td>
<td>296</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR474</td>
<td>Post-1996</td>
<td>M</td>
<td>299</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR476</td>
<td>Post-1996</td>
<td>F</td>
<td>299</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR463</td>
<td>BSE</td>
<td>M</td>
<td>148</td>
<td>Recumbency</td>
<td>+/+/+</td>
</tr>
<tr>
<td>PR443</td>
<td>BSE</td>
<td>F</td>
<td>175</td>
<td>Recumbency</td>
<td>+/+/+</td>
</tr>
<tr>
<td>PR442</td>
<td>BSE</td>
<td>F</td>
<td>234</td>
<td>Arthrosis</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR441</td>
<td>BSE</td>
<td>M</td>
<td>294</td>
<td>Killed at termination of study</td>
<td>–/–/– *</td>
</tr>
<tr>
<td>PR444</td>
<td>BSE</td>
<td>F</td>
<td>294</td>
<td>Killed at termination of study</td>
<td>–/–/– *</td>
</tr>
<tr>
<td>PR462</td>
<td>BSE</td>
<td>M</td>
<td>297</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR460</td>
<td>BSE</td>
<td>F</td>
<td>297</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR440</td>
<td>BSE</td>
<td>M</td>
<td>298</td>
<td>Killed at termination of study</td>
<td>–/–/– *</td>
</tr>
<tr>
<td>PR620</td>
<td>NZ-brain</td>
<td>F</td>
<td>17</td>
<td>Fracture of right femur</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR593</td>
<td>NZ-brain</td>
<td>F</td>
<td>84</td>
<td>Spinal abscess</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR596</td>
<td>NZ-brain</td>
<td>M</td>
<td>218</td>
<td>Arthritis</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR591</td>
<td>NZ-brain</td>
<td>F</td>
<td>275</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR594</td>
<td>NZ-brain</td>
<td>F</td>
<td>275</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR598</td>
<td>NZ-brain</td>
<td>M</td>
<td>275</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR599</td>
<td>NZ-brain</td>
<td>F</td>
<td>275</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR621</td>
<td>NZ-brain</td>
<td>F</td>
<td>276</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR623</td>
<td>NZ-brain</td>
<td>F</td>
<td>276</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
<tr>
<td>PR627</td>
<td>NZ-brain</td>
<td>M</td>
<td>276</td>
<td>Killed at termination of study</td>
<td>–/–/–</td>
</tr>
</tbody>
</table>

N/A….Not applicable, HP…Histopathology
F……..Female, M……..Male (castrated)

* Neuropathological examination extended to representation of all brain regions
Table 3. Presence of selected TSE-like clinical signs or combination of signs observed at the pre-cull assessment in the transmission study

<table>
<thead>
<tr>
<th>Signs</th>
<th>Groups</th>
<th>( P ) value by group comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Repeatable over-reactivity to menace testing</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Apprehension towards observer</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Repeatable over-reactivity to neck prick</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Stiff gait</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Ataxia</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Apprehension, over-reactivity &amp; ataxia/ stiff gait</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Apprehension &amp; over-reactivity</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Apprehension &amp; ataxia/ stiff gait*</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Over-reactivity &amp; ataxia/stiff gait*</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

The numbers in each group refer to the number of animals displaying this particular sign or combination of signs.

ns = not significant (\( P > 0.05 \)).

Group A = NZ brain, includes pig that was not inoculated (N=10)
Group B = BSE (N=9)
Group C = Pre-1996 group (N=10)
Group D = Post-1996 group (N=10)

* Signs associated with a prion disease; the one animal in group D with multiple abscesses is listed for completeness although a prion disease was not suspected.
Figure 1a-i. Comparison of the immunolabelling observed in three different pigs using two different antibodies

Coronal section at the level of rostral midbrain from a negative, non-inoculated animal (PR459 = PG1737/04) treated with 2G11. Note the absence of any non-specific background.
Serial coronal sections at the level of rostral midbrain from a TSE-negative clinical suspect, which had been inoculated with BSE (PR442 = PG1348/05). In Figure b IHC was performed using antibody 2G11 while in Figure c the antibody L42 was applied. Note the heavy intraneuronal non-specific labelling in the presence of L42.
IHC of the rostral midbrain from a BSE positive pig (PR443 = PG0954/03) using mAb 2G11. Specific immunolabelling is evident in the neurons (intraneuronal) and the neuropil.
IHC of the rostral midbrain from the same BSE positive pig (PR443 = PG0954/03) using mAb L42. This antibody produces heavy non-specific background against which it is difficult to appreciate the degree of specific labelling.
IHC of the rostral midbrain from the same BSE positive pig (PR443 = PG0954/03) using mAb L42. Attempts to decrease the non-specific background associated with L42 resulted in a concomitant reduction of specific signal. Compare Figure i with Figures e and g, and Figure h with Figures d and f.
Figure 2. Western immunoblot of a sample of the caudal medulla of pigs from each group.

1. Biotinylated molecular mass marker
2. BSE group (positive) PR463 = PG0033/03
3. BSE group (positive) PR443 = PG0954/03
4. NZ–brain (negative) PR591 = PG1328/05
5. Pre-1996 group (negative) PR469 = PG1331/05
6. Post-1996 group (negative) PR476 = PG1342/05
7. BSE group (negative, clinical suspect) PR442 = PG1348/05
8. Non-challenged pig (negative) PR459 = PG1737/04
9. Bovine BSE +ve
10. Ovine Scrapie +ve
11. Biotinylated molecular mass marker

VLA Hybrid technique (discriminatory WB) using two different monoclonal antibodies, 6H4 (A) and P4 (B), to distinguish scrapie (signal with both antibodies) from BSE (no signal with P4, lower molecular mass of the un-glycosylated band)
compared to scrapie with 6H4).