Rev. sci. tech. Off. int. Epiz., 2003, **22** (1), 283-296

The potential for transmissible spongiform encephalopathies in non-ruminant livestock and fish

D. Matthews [1] & B.C. Cooke [2]

(1) Transmissible Spongiform Encephalopathy (TSE) Programme Manager, Veterinary Laboratories Agency, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom
(2) 1 Jenkins Orchard, Wick St Lawrence, Weston Super Mare, North Somerset, BS22 7YP, United Kingdom

Summary
Pigs and poultry in the United Kingdom have undeniably been exposed to the bovine spongiform encephalopathy (BSE) agent. They consumed the same ruminant protein that gave rise to the BSE epidemic in cattle, but there has been no evidence of an epidemic in these species. Experimental investigations have shown pigs to be susceptible to infection by multiple parenteral challenge, but resistant to oral exposure with BSE-infected cattle brain. Current but incomplete evidence suggests that they are also resistant to oral challenge with sheep scrapie. Studies in domestic chickens indicate that they are resistant to both parenteral and oral challenge. Unfortunately, no published data exists on the susceptibility of fish to infection. Incidental findings in the brains of unexposed pigs are described that could otherwise give rise to concerns about the presence of a transmissible spongiform encephalopathy in pig populations around the world.

Keywords
Chicken – Fish – Meat-and-bone meal – Pig – Poultry – Prion – Prion protein – Transmissible spongiform encephalopathy.

Introduction

The recognition of bovine spongiform encephalopathy (BSE) in domestic cattle in the United Kingdom (UK) in 1986 inevitably led to concerns about the potential risk to non-ruminant livestock (49). Although the initial focus was on the identification of the causal agent of BSE and confirmation that the disease was transmissible (20), research rapidly investigated the likelihood that pigs and poultry might also be susceptible to infection.

Initial epidemiological investigations into the source of BSE identified the likely vehicle to be the consumption by cattle of rendered animal protein of ruminant origin (51). With time, the decline of the epidemic following the implementation of measures to remove ruminant protein from cattle feed has confirmed that hypothesis. This is distinct from the debate about whether the actual origin of the agent was ovine or bovine. While there has been speculation about whether BSE arose spontaneously in bovines, rather than from transmission of sheep scrapie to cattle, the ban dealt with the vehicle of transmission, irrespective of origin.

The same investigations also identified the fact that consumption of meat-and-bone meal (MBM) must have led to significant exposure of the pig and poultry populations in Great Britain to the causal agent of BSE. This paper reviews the history of the use of animal-derived proteins in livestock feed in Great Britain, especially with respect to pigs and poultry. The authors then consider the limited information on the genetic susceptibility of non-ruminant species to transmissible spongiform encephalopathies (TSEs). Finally, they summarise investigations into the experimental transmissibility of BSE to pigs and poultry, some of which, because of their long-term nature, have not yet been formally published. The potential for inactivation of TSEs by rendering processes is not considered, as this is dealt with by Taylor and Woodgate in this book (47). The absence of a natural TSE in such species is only addressed

in terms of their susceptibility to infection, rather than through any selective processing which may have proved protective. Such a scenario is unlikely.

Historical use of meat-and-bone meal

This brief review is intended to demonstrate the general acceptability and use of MBM not only in animal feeds in the UK, but also throughout the world. Indeed the nutritional value as a source of both amino acids and minerals indicates why this raw material has become such an important ingredient of feed for poultry, pigs and fish (Table I).

Table I
Nutrient contents of animal protein materials based on data from leading feed compounders in the United Kingdom

Nutrient	Meat-and-bone meal*		Meat meal	Blood meal	Feather meal	Poultry offal
	Low protein	High protein				
Oil/fat (%)	9.0	10.0	10.0	2.0	5.0	20.0
Crude protein (%)	40.0	50.0	55.0	80.0	85.0	60.0
Total lysine (%)	2.2	2.8	3.1	7.0	1.4	2.2
Total methionine (%)	0.5	0.6	0.7	1.0	0.3	0.7
Total methionine and total cystine (%)	0.8	1.0	1.1	2.4	2.2	2.1
Total threonine (%)	1.3	1.6	1.7	3.8	3.6	2.0
Total tryptophan (%)	0.2	0.3	0.4	1.1	0.4	0.5
Calcium (%)	12.2	9.8	7.5	0.3	0.5	1.0
Phosphorous (%)	5.9	4.8	4.0	0.3	0.3	0.7
Salt (%)	0.8	1.2	1.4	0.9	0.3	0.7
Digestible energy pig (MJ/kg)	10.0	11.0	11.3	12.7	11.5	14.5
Metabolisable energy poultry (MJ/kg)	8.8	9.5	10.1	12.0	10.9	14.5

* The nutrient values of meat and bone vary according to the proportion of fat and bone tissue included with other offal prior to rendering

Use of meat-and-bone meal in pig and poultry feeds before the occurrence of bovine spongiform encephalopathy

In 1915, William Goodwin, of the University of London, published 'The scientific feeding of animals,' an English translation of an earlier work by Kellner (28). This refers to the earliest record of the use of meat by-products in animal feeds by Liebig in 1865. A description is given of meat meal (MM) produced by the 'Liebig method'. The new 'competing material' is stated to have been derived from carcass material heated by super-heated steam in large drums containing revolving knives, the heating and mincing continuing until a dry powder remained. Glue and fat drawn from the drums were obtained as by-products. This is clearly a description of the early days of the rendering process, and the dry powder produced was an early type of MBM. In 1908, Kellner reported that this carcass meal had already been in use as an animal feed for a few years but that horses, oxen and sheep either refused to eat it or did so reluctantly. Swine were reported to consume the carcass meal readily.

The acceptability of MBM as an animal feed ingredient in the UK in the first quarter of the century is best demonstrated by the description of the material as a feedingstuff in the Fertiliser and Feedingstuffs Act in 1926 (35). This inclusion followed a report to parliament in 1924 entitled 'Report of the Fertilisers and Feedingstuffs Advisory Committee' (33), which recommended that raw materials used in compound feeds should be listed in new legislation. A further report in 1925 recommended the materials be listed in the Schedules to legislation and included 'MBM', 'bone flour' and 'MM' (34). Meat-and-bone meal was defined as the product of drying and grinding of bone, flesh and flesh fibre to which no other matter has been added. In describing American feed materials and feeding practice, Morrison referred to rendered products comprising MBM with a protein content of 40% to 60% and an oil content of 1% to 10% (39). The meat by-products were reported to be fed to pigs at inclusion rates of 9% to 10% in pig weaner diets and 6% to 8% in finisher diets. Dried blood meal was reported as being particularly useful for feeding to young pigs as a skim milk substitute.

The Fertilisers and Feedingstuffs Regulations 1932 (36) introduced a revised definition for MBM, requiring that the feedingstuff contain not less than 40% protein and not more than 4% salt, a definition that remained in force until the UK joined the European Community in 1974.

Numerous references in scientific literature during the 1930s and 1940s indicate that the use of MBM, MM and blood meal in diets for pigs and poultry was common practice in many countries. In the UK during, and immediately after, the Second World War the manufacture of animal feeds was controlled essentially by Orders passed under the powers of Regulation 55 of the Defence (General) Regulations 1939 (1). These orders serve to give an indication of trends in the use of MBM during the 1940s.

The Feedingstuffs (Regulation of Manufacture) Order 1942 (2) defined a group of raw materials as 'animal protein rich substances meaning any substance other than dried blood derived from any animal or fish and which contains not less than 40% protein'. Clearly meat-and-bone would fall within this definition. The definition of compound animal feed is given as 'a mixture for livestock containing not less than 5% of four ingredients listed in the Schedule'. The Schedule includes 'feeding MM (including feeding MBM)' and 27 other ingredients. The Order defines protein concentrates used for mixing with cereals to produce final feeds as containing a minimum of four ingredients from the Schedule, but does not

lay down minimum inclusion levels for them. There are eleven ingredients listed in the Schedule for Concentrates, which include 'feeding MM (including feeding MBM)'. Thus feeding MBM was well recognised as being an ingredient in compound feeds during the war.

Later revision introduced the concept of minimum or maximum inclusion levels of animal-derived protein-rich substances in a number of national feeds, including some for pigs and poultry (3). Clearly, at times during the 1940s, feed compounders were obliged by law to incorporate MBM in some products for monogastric animals. Although by December 1947 (4), minimum inclusion levels were no longer specified, 'feeding MM (including MBM)' continued to be listed in the schedules of later legislation for use in both compound feed and concentrates (5).

The post-war era saw rapid expansion of the layer, broiler, turkey and pig industries in many countries in Europe, North America and Australasia. Meat meal, MBM, blood and feather meal became well established as sources of amino acids, energy and minerals in commercial feed production for farm animals. As the broiler industry in particular expanded, the production of poultry offal meal also increased quite dramatically. This animal protein source came into common use by the late 1960s. Scientific research into the use of these animal proteins continued through the 1960s and 1970s, and many reports were published in scientific journals and books. King referred to the typical inclusion of 2.5% MBM in rations for starter and growing fowl, 5% to 6.25% MM for layers and 2.5% to 5% MBM for turkeys (30). Typical maximum inclusion rates of these feed materials in commercially produced animal feeds in the UK by the late 1960s are summarised in Table II (30).

Similar inclusion rates were used in diets in Europe and North America and although the predominant animal protein was MBM (protein content of 40 to 50%), in some regions, MM (protein content of 50% to 55%) was used. Inclusion rates of MBM were fairly constant, but on occasions when supply and demand led to an imbalance between the cost of extracted soya and MBM, inclusion rates were reduced. The use of blood, feather and poultry offal meals was more variable, although many integrated poultry businesses adopted the constant use of poultry offal meal produced from the by-products of their own processing business. Throughout the UK and most of Europe, the MBM used would have been produced from offal arising from pig, ruminant and, to a lesser extent, poultry meat production. As a result of these feeding practices, pig products would have been recycled to pigs and poultry products to poultry. Both pigs and poultry would have been fed on ruminant-derived animal proteins.

During the 1970s, higher nutrient density of animal feed delivered improved growth rates per unit of feed consumed. At the same time, there was a growing appreciation of the importance of quantities of, and balance between, essential amino acids in both pig and poultry feeds to ensure efficient growth and performance. This resulted in a greater dependence on feed materials with a comparatively high content of digestible amino acids. The choice of raw materials, from synthetic to naturally derived amino acids of animal origin, was driven by their relative costs.

Table II
Typical maximum inclusion rates of various animal proteins in pig and poultry feeds at the end of the 1960s and in the 1980s, based on data supplied by leading feed compounders in the United Kingdom

Feed type	Meat-and-bone meal [a]		Meat meal [a]		Blood meal [b]		Feather meal [b]		Poultry offal [b]	
	1960s	1980s	1960s	1980s	1960s	1980s	1960s	1980s	1960s	1980s
Pig weaner	2.5	2.5	2.5	2.5	0	0	0	0	0	0
Pig grower	6.0	7.5	6.0	7.5	1.0	1.0	1.0	2.0	0	0
Pig finisher	7.5	8.0	7.5	8.0	1.0	1.0	2.0	2.0	0	0
Pig breeder	7.5	8.0	7.5	8.0	1.0	1.0	1.0	2.0	1.0	1.0
Replacement layer starter	5.0	5.0	5.0	5.0	0	0	0	0	0	0
Replacement layer grower	6.0	8.0	6.0	8.0	1.0	1.0	1.5	1.5	1.0	1.0
Layer	7.0	8.0	7.0	8.0	1.0	1.0	1.0	1.0	0	0
Breeder (layer/broiler)	7.0	8.0 [c]	7.0	8.0 [c]	1.0	1.0	1.0	1.0	0	0
Broiler starter	5.0	5.0	5.0	5.0	0	1.0	0	0	1.0	1.0
Broiler grower	5.0	5.0	5.0	5.0	1.0	1.0	1.0	1.0	2.0	2.0
Broiler finisher	5.0	5.0	5.0	5.0	1.0	1.0	1.0	1.0	2.0	2.0
Turkey starter	5.0	5.0	5.0	5.0	0	1.0	0	0	1.0	1.0
Turkey grower	5.0	5.0	5.0	5.0	1.0	1.0	1.0	1.0	2.0	2.0
Turkey finisher	6.0	7.5	6.0	7.5	1.0	1.0	1.0	1.0	2.0	2.0
Turkey breeder	5.0	5.0 [c]	5.0	5.0 [c]	1.0	1.0	1.0	1.0	2.0	2.0

a) These materials would have a combined maximum equivalent to that of meat-and-bone meal
b) These materials would have a combined maximum equivalent to the highest inclusion of any individual
c) By the late 1970s/early 1980s, these materials were often excluded from breeder feeds as part of salmonella control programmes

Towards the end of the 1970s, a number of retailers and integrated poultry companies (companies that breed, rear, fatten and slaughter poultry) became increasingly concerned about the possible role of MBM in introducing *Salmonella* spp. into their flocks. This resulted in the voluntary withdrawal of MBM from poultry feeds for breeders in particular.

Compound feed production for sale in the UK increased between 1950 and 1970 (9). In the case of pig feed, production rose from approximately 1 million to 2.5 million tonnes per year. Poultry feed production rose from 1.3 million to 4 million tonnes per year in the same period. In the years 1970 to 1990, pig feed tonnage for sale remained fairly static, at about 2.3 million tonnes per year, whilst poultry feed tonnage for sale slowly declined. In addition, many pig farmers produced their own feed and large integrated poultry operations began or increased production of their own feed. Reasonably, total pig feed consumption is assumed to have exceeded 3.5 million tonnes per year and total poultry feed to have exceeded 4.5 million tonnes per year during this period.

An average inclusion rate of 5% MBM in pig feeds in the 1980s would thus indicate the usage of in excess of 175,000 tonnes per year of meat-derived products in pig diets in the UK. At an average inclusion rate of 5% meat-and-bone in poultry feeds, the comparable usage would be in excess of 225,000 tonnes per year.

Use of meat-and-bone meal in pig and poultry feeds after the occurrence of bovine spongiform encephalopathy

The advent of BSE in the UK and the subsequent association of the disease with the use of ruminant-derived MBM in ruminant feeds had a marked effect on the use of animal proteins in feed for monogastric animals in the UK and Europe.

The ban on the use of ruminant protein in ruminant feed (6) raised concern about inter- and intra-species recycling, especially within the poultry industry. The question was raised as to whether BSE could be transmitted to poultry and whether the use of poultry offal or feather meal in poultry feed could have propagated infectivity within the poultry industry. As a result, some feed companies discontinued using poultry offal and feather meal in poultry feeds from the late 1980s. Indeed, between 1990 and 1996, some feed companies stopped using animal proteins altogether, other than fish meal and milk products, in feeds for pigs and poultry. Others continued to use these ingredients until the use of mammalian MBM in livestock feed was banned in 1996 (8).

Despite the 1996 ban in the UK, the feeding of mammalian MBM to pigs and poultry remained legal in other countries of Europe. Nevertheless, sentiment and market forces contributed to a marked decline in the use of this feedingstuff in the last few years of the 1990s. Following the 1994 European ban on the use of mammalian protein in ruminant feeds (15), many feed mills that manufactured both ruminant and non-ruminant animal feeds, ceased using animal proteins in any feeds.

Since January 2001 (16, 17, 18, 19), use of all processed mammalian protein in feeds for farmed animals has been banned throughout the European Union (EU), with periodic adjustments, but these materials continue to be used in pig, poultry and indeed ruminant feeds in other parts of the world. Throughout the EU, vegetable proteins, especially soya meal and synthetic amino acids, have replaced animal proteins. Calcium carbonate and phosphates are preferred sources of minerals. As animal-derived proteins also provided essential vitamins, such as vitamin B12, which is not found in vegetable protein materials, additional supplementation with vitamins has also had to follow the withdrawal of animal products.

Use of meat-and-bone meal in fish feeds

Historically, animal proteins of many types have been used in feeds for fish culture. Early attempts to develop complete moist diets for fish included the use of fresh and frozen wet fish, other aquatic animals (often live), processing waste from domestic animals, waste human food, eggs and even live insects. Almost anything was tried in attempts to maintain and grow fish satisfactorily and one of the traditional animal proteins used was fresh liver.

Moist diets were however perishable, difficult to store, messy to prepare and generally inconvenient to use. In addition, they could only be used locally and did not readily lend themselves to manufacture in large quantities as fish production increased. Many of the problems were overcome by the development of dry diets, although wet feeding has not completely disappeared. Many new marine species that are being developed for farming, such as rotifers and artemia, are still fed live wet feed in their juvenile stages.

The first dry fish feed pellets, developed in the 1950s and 1960s, were produced by pressing the ingredients through ring dies in the same way that many animal feeds are still produced today. Typical meat proteins used in these diets were liver meal, MM, MBM, blood meal, feather meal and poultry offal meal. These early dry feeds had a high protein and low energy content and were ideal ingredients alongside fishmeal.

By the mid 1980s, research into fish nutrition and the introduction of cooker extrusion production technology allowed the development of feeds with higher energy and reduced protein content. At this time, many of the traditional animal proteins were being diverted into the pet food industry, leaving only blood meal, MBM and feather meal for use in fish feeds. In general, MBM was confined to the lower energy trout feeds.

Typical inclusion rates of animal proteins in fish feeds in the UK in the period leading up to 1989 were 5% MBM or MM, 4%

blood meal, 6% to 7% feather meal and 3% to 5% poultry offal meal. The maximum inclusion rate for each ingredient was 10%, except for blood meal at 8%. In the late 1980s, total fish feed production in the UK was in the region of 75,000 tonnes per year. This would indicate the use in fish feed of about 3,750 tonnes of MBM or MM and about 11,250 tonnes of other animal proteins per year.

In 1989, in response to media publicity and retail concerns about potential cross-species transmission of BSE, all three UK-based fish feed manufacturers agreed to voluntarily remove materials of bovine and ovine origin from all fish feed formulations. In practice, this meant the complete removal of MBM and changes to sources of blood meals. Thus, together with fishmeal and crustacean meal, blood meal and feather meal continued to be used, but blood meal was specified as being of porcine or avian origin only. In Europe, however, MBM continued to be used in the early 1990s at levels somewhat higher than those used in the UK during the 1980s.

This situation continued until March 1996 when the probable link between BSE and variant Creutzfeldt-Jakob disease (vCJD) was announced. Again, media interest resulted in pressure from retailers for the exclusion of blood and feather meals from fish diets. This led to progressive, voluntary withdrawal of blood and feather meals before the end of 1996. Since then, the sole source of animal proteins in UK-produced fish feeds has been fish meal and crustacean meal.

Experimental investigations of susceptibility to bovine spongiform encephalopathy

Given that epidemiological investigations identified the significant use of MBM of ruminant origin in feeds for both pigs and poultry, both populations were therefore inevitably exposed to BSE to a significant degree. In time, molecular studies, involving *in vitro* conversion of prion protein (PrP) and comparison of PrP gene sequences, would be developed. These can now be used as potential indicators of susceptibility to infection across species barriers. Nevertheless, key studies remain the determination of the transmissibility of the BSE agent to pigs or poultry by parenteral or oral exposure to BSE-infected bovine brain.

Susceptibility of pigs to parenteral challenge with bovine spongiform encephalopathy

The first study was initiated in 1989 and outline protocols were described by Dawson *et al.* in 1991 and 1994 (13, 14). Full protocols have been described by Wells *et al.* (50). Ten Landrace × Large White piglets were inoculated by multiple parenteral routes at one to two weeks of age with a 10% homogenate of BSE-infected bovine brainstems. Each piglet was injected with 0.5 ml intracranially, 8 ml to 9 ml intraperitoneally, and 1 ml to 2 ml intravenously. A group of control piglets was similarly inoculated with saline. The approximate titre of the inoculum was 10^5 intracerebral (i.c.) infectious dose $(ID)_{50}$ mouse infectious units per gram (21). Although challenge and control pigs were housed separately, all pigs subsequently received the same commercial pig ration.

This study, using a large parenteral exposure, was conducted to determine whether BSE could be transmitted to pigs using the most effective routes of transmission of TSEs. Clinical signs were monitored. An interim kill was planned at two years post challenge, and, in the absence of clinical disease, the study was to be terminated at five years. At interim and final kills, or following intercurrent death, necropsies were conducted, pathological changes recorded and a range of fixed and frozen tissues collected, the latter for bioassay.

The successful transmission of BSE to one of the challenged pigs was first reported in 1990 (12). Transmission was confirmed at 74 weeks post inoculation. This led to immediate exclusion of tissues that are currently recognised as specified risk materials (bovine brain, spinal cord, tonsil, thymus, spleen and intestine) from the food of domestic livestock and domestic pets (7). This is still the only measure specifically introduced to protect non-ruminant animals in the UK, although it will have been reinforced by the wider feed ban of 1996 (8).

When the study was concluded, BSE had clearly been successfully transmitted to at least seven of the ten challenged pigs (23, 43, 50). Five animals exhibited clinical signs, with an incubation range of 69 to 150 weeks, while the remaining two were identified preclinically following post-mortem examination after slaughter of clinically normal pigs at 24 months post challenge.

Clinical signs in all affected pigs were similar to those reported by Dawson *et al.* (12). The clinical course was characterised by initial behavioural changes, including mild aggressive behaviour towards attendants and mild ataxia. Early signs also included increased timidity where previously dominant and aggressive, increased vocalisation, mild hypermetria of forelimbs, hind limb weakness and abnormal carriage of the ears. Progression of clinical signs from the earliest subtle behavioural changes to overt clinical disease and euthanasia took between five and thirteen weeks. Ataxia progressed to weakness, falling and recumbency. All control animals remained free of any evidence of transmission (50).

Histopathological examination of the brain revealed changes indicative of spongiform encephalopathy in seven of the exposed group. The changes were severe and extended throughout most regions of the brain in clinically affected pigs. Lesions were more variable and less severe in the clinically normal pigs killed at 24 months post inoculation, while the

clinically affected pig killed at 74 months post inoculation showed lesions of intermediate severity.

Vacuolar changes were most concentrated on the basal nuclei and diminished in intensity caudally, being least severe in the caudal brainstem. Prion protein distribution in the central nervous tissue, as visualised by immunohistochemistry, was typical of disease specific accumulations in other species. Details of the neuropathology in the study have been published in full elsewhere (43). Although all pigs were examined for the presence of scrapie-associated fibrils (SAF), these were only detected in the first pig reported (12).

Bioassay in C57BLJ6 mice of a range of tissues sampled at interim kills of healthy pigs, following euthanasia due to clinical disease or at termination, identified infectivity only in the brain, spinal cord, stomach, jejunum, distal ileum and pancreas of exposed pigs (50). No infectivity was detected in other tissues that were assayed, including the spleen, thymus or mesenteric and popliteal lymph nodes. While the incidence of disease in assay mice inoculated with central nervous system tissues indicated moderate to high levels of infectivity, the incidence of disease in mice inoculated with the positive alimentary tissues indicated low to limiting dilution levels of infectivity. No evidence of infectivity was found in unexposed control pigs.

Susceptibility of pigs to oral challenge with bovine spongiform encephalopathy

Given the likelihood that natural exposure of pigs to BSE would have been by the oral route, a further study was established in parallel to the previous one. For this experiment, recipient pigs were used from litters of five sows, representative of five popular breed types (hybrids) in the UK. The piglets were fed a ration free of MBM. After weaning at seven to eight weeks, a castrated male and a female from each litter were assigned to challenge and control groups.

The design of the study aimed to replace the maximum daily intake of MBM for the appropriate age of pig with the equivalent dry matter of BSE-infected brain pool on three successive occasions. Mimicking the multiple low dose exposure that may have occurred naturally was not feasible, or indeed desirable in an initial challenge study. The pigs in the challenge group were therefore fed approximately 4 kg of brain homogenate on each of three occasions, at one to two week intervals. Assuming consumption of equal amounts, the total amount consumed by each pig was therefore 1.2 kg. Although the homogenate was fed on the floor, feeding was observed to confirm that each pig consumed a substantial proportion of the total. The titre of the brain pool used in the oral challenge was estimated at $10^{2.4}$ i.c./intraperitoneal (i.p.) ID_{50} mouse infectious units per gram, considerably lower than that used in the parenteral challenge exposure study.

As with the parenteral challenge, an interim kill was planned at two years post exposure, but the terminal kill was extended to seven years post challenge in the absence of evidence of clinical disease. Again, neuropathological examinations and infectivity assays were conducted for all pigs.

In contrast to the first study, there was no evidence of successful transmission of BSE in the form of clinical signs or pathological changes indicative of a spongiform encephalopathy (50). No evidence of infectivity was detected in any of the tissues inoculated into C57BLJ6 or RIII mice. One possible explanation for this result is that the infectious dose with which the pigs were challenged was insufficient to produce infection. In fact, the dose given was slightly greater than that given parenterally, at approximately $10^{5.5}$ mouse infectious units compared with 10^5 units parenterally. Despite the relatively low starting titre of the inoculum, the experimental oral exposure in this study is estimated to be of the order of 50,000 times greater, on each occasion of dosing, than would occur naturally though feed on farm (50). The result would therefore be consistent with evidence that the oral route is less efficient than parenteral routes (29), and with the absence of evidence for natural infection of pigs occurring through multiple low dose exposure via feed.

Susceptibility of pigs to oral challenge with sheep scrapie

Following the establishment of the oral challenge study with BSE, another experiment began in 1993 in which piglets were exposed orally to ovine brain homogenate from scrapie-affected sheep. This study was not preceded by parenteral challenge with scrapie. The objective was simply to compare the results with those of the BSE oral challenge.

The selection of recipient piglets, representative of breed types in Great Britain, and the design of the study, were identical to those for the oral challenge study with BSE. The challenge group (on this occasion, 12 piglets aged seven to eight weeks) was floor-fed with homogenate (4.8 kg) on each of three occasions at weekly intervals. An aliquot of the scrapie brain challenge material was titrated in mice and the titre estimated to be $10^{3.69}$ i.c./i.p. ID_{50} mouse infectious units per gram. The average dose received was therefore greater than in the BSE challenge study, at approximately $1.2 \times 10^{6.69}$ mouse infectious units.

Twelve control piglets were also taken from the same litters and these received normal rations only. Culls of four challenged pigs and four controls were planned at two, five and seven years post exposure, or at intervening clinical onset. A range of tissues were again collected for bioassay and histopathological examination.

One exposed pig was killed due to intercurrent disease 12 months after exposure. No significant lesions were observed in the brain on histopathological examination. At two years post challenge, four control and three exposed pigs, all

clinically normal, were culled. Histopathological examination of their brains revealed no significant lesions. Bioassays of tissues in mice have revealed no evidence of infectivity.

At 28 months post exposure, three pigs were displaying mild clinical signs, including fine tremor of the ears. One of these subsequently developed postural difficulties and fasciculation of the facial muscles, which became a constant sign. This pig continued to deteriorate and was culled at 34 months post challenge. There was no evidence of TSE-like spongiform change in the brain. Of the other two pigs, one returned to normal behaviour and the other died 72 months after challenge. At necropsy, abdominal neoplasia was detected. Histopathological examination of the brain from this pig, along with that of two control pigs killed due to intercurrent disease 44 and 71 months post challenge, revealed no significant lesions. Interestingly, a syndrome in Landrace type pigs, 'Landrace tremor', has been recorded historically but is poorly documented.

Three challenge pigs and three control pigs killed five years after challenge also revealed no significant lesions. The remaining three challenged and three control pigs were killed 84 months post challenge, and again did not reveal evidence of transmission.

None of the bioassay studies have revealed any evidence of infectivity. Those conducted on tissues collected two years post exposure are complete. Assays on tissues collected at seven years post challenge remain incomplete, but there has been no evidence of transmission at a minimum of 440 days post inoculation.

Incidental findings in pigs

An interesting finding in the parenteral challenge study was the presence of varying degrees of vacuolation of the superficial layers of the grey matter of the rostral colliculi of both challenged and control pigs (43). The vacuoles in control pigs were variable in size, but indistinguishable, except in number, from those found at the same site in affected animals. The vacuoles did not however extend into the deeper layers of the rostral colliculi or into other areas of the mid-brain or diencephalon. Vacuolation of neurones of the dorsal nucleus of the vagus and of the hypothalamus, in the internal capsule and occasionally other white matter tracts, were also seen in control animals. For a full description of the pathology, readers are referred to the primary publication (43).

The origin of this vacuolation was unclear, but considered to be normal, as occasionally reported for cattle (38), sheep (53) and dogs (32). Subsequent examination of archived porcine brain material from other parts of the world supports this view. To clarify the situation and hopefully confirm this interpretation, further studies were initiated to determine whether or not such lesions represent the presence of an infectious agent. Rostral colliculi from healthy pigs born before and after the 1996 feed bans (8), together with brain from healthy pigs sourced from a country that has never experienced BSE, have been inoculated intracranially, intravenously and intraperitoneally into two-week-old Large White piglets. Another group was inoculated with bovine BSE as a positive control. The dosing protocol followed that used in the first BSE parenteral challenge study. All groups comprised ten pigs, and will be observed for five years unless preceded by intervening death. The study is currently 28 months post challenge, and the only indication of transmission is in the positive control group where possible early clinical signs are detectable (S.J. Ryder, personal communication).

Susceptibility of poultry to bovine spongiform encephalopathy

In June 1990, studies were initiated to examine the transmissibility of BSE to domestic fowl. Twelve day-old chicks were inoculated intracerebrally with 50 µl of 10% bovine brainstem homogenate from BSE-affected cattle. Then, at two weeks old, they were also inoculated intraperitoneally with 1 ml of the homogenate. A further group of eleven chicks was challenged orally at four, five and six weeks of age with pooled brainstem from BSE-affected cattle. Each bird received 5 g of pooled brain tissue by deposition into the distal oesophagus/crop using a syringe attached to a length of flexible plastic tubing. Fourteen chicks, reared and maintained similarly, were inoculated intracerebrally and intraperitoneally with saline only to serve as controls for both the parenteral and dietary challenge studies. The starting titre of BSE inocula cannot be reported at the time of writing as titration is still in progress.

All of the birds were maintained on a ration free of MBM and housed singly in cages akin to battery housing. They were clinically monitored up to five years post inoculation when they were killed and necropsy examinations conducted. Selected tissues were sampled for histopathological examination and retained for potential bioassay. Birds showing clinical disease or dying before the planned kill were examined similarly. There was no evidence of transmission during the study as indicated by the appearance of a consistent clinical disorder confined to challenged birds and the histopathological examination of the brain revealed no significant lesions in any of the birds.

During the course of the study, a number of birds were lost due to intercurrent disease. Most of the losses were attributed to incidental causes that were not manifested as neurological disorders and occurred to a similar extent in challenged and control birds. A clinical neurological syndrome, loosely termed 'motor disturbance', was however noted from 22 months to the terminal kill at 60 months post inoculation in male birds, but only in the challenged groups. Four parenterally and four orally challenged birds were affected. Although the clinical presentation of this disorder was slowly progressive, no

significant degenerative pathology was identified in the brain, spinal cord, sciatic nerve or skeletal muscle tissues of these birds. This syndrome, as far as can be ascertained, has not been reported or experienced in commercially kept domestic fowl. However, the life span of fowl under farmed conditions is usually no more than 18 months, less than the age of the fowl presenting this syndrome. Interpretation of this observation was also confounded by the survival rates of the male control birds. Insufficient male control birds survived to terminal kill to determine whether or not the controls were susceptible and to eliminate the possibility that the disorder was simply an age- or husbandry-related disorder. A substantial proportion of the female birds, both challenged and controls, survived to termination of the study and did not develop disease.

Sub-passage of material from challenged fowl

To investigate the motor disturbance syndrome further, 50 µl of a 10% suspension of a pool of nervous system tissues (frontal cortex of brain and sciatic nerve) from birds clinically affected with the 'motor disturbance' syndrome were inoculated intracerebrally into groups of ten day-old chicks. In one group, the inoculum was derived from birds of the primary parenteral challenge study and in another group, the inoculum came from birds of the primary oral challenge study. To provide appropriate control groups, a group of day-old chicks was inoculated with a comparable pool of nervous system tissues derived from clinically normal, saline inoculated birds from the original study and another group was inoculated with saline only. In addition, nervous system tissues (frontal cortex and sciatic nerve) from the clinically affected birds were subject to mouse bioassay in RIII, C57Bl, VM, IM and C57Bl × VM mice.

The sub-passage in poultry is now complete at 60 months post challenge. Mouse bioassay is also complete. Neither line of sub-passage detected infectivity in the chickens that were the subject of primary exposure to BSE. Furthermore, the clinical syndrome noted in the primary passage was not replicated on sub-passage. Histopathological examination of brains of mice and chickens did not reveal pathological changes that were indicative of infection. In addition, a range of antibodies to mammalian species PrP was used to attempt PrP immunostaining in the brains of challenged and control chickens, but no specific staining was detected. However, this study was compromised by the absence of an avian positive control model and the unavailability of antibodies specific to, or known to cross-react with, an avian homologue of mammalian PrP. The appropriateness of the mammalian PrP antibodies employed is debatable given the lack of homology between mammalian and avian PrP (52).

Susceptibility of fish to bovine spongiform encephalopathy and scrapie

No attempt has been made to conduct experimental challenges of fish in the UK. Bovine spongiform encephalopathy-infected material has been provided to one European institute for experimental challenge of fish in a project funded by the European Commission. Experimental challenges with laboratory adapted strains of scrapie are understood to serve as controls. As the work is currently in progress, no results are available at the time of writing.

Genetic susceptibility to transmissible spongiform encephalopathies

Across the spectrum of the TSE (or prion) diseases, a single gene encoding a normal membrane protein, PrP, is pathogenetically or aetiologically linked to disease occurrence. The association between PrP genotype and potential susceptibility to infection with TSEs, and especially scrapie, has been the subject of in-depth study (25, 26). The principle that genotype determines susceptibility has also been investigated *in vitro* (10, 31, 42) and although conforming with *in vivo* studies, *in vitro* conversion of normal PrP into an infectious molecule has yet to be shown. Nevertheless, the degree of homology between PrP sequences of donors (source of TSE) and recipients (challenged/exposed animals) apparently has a significant impact on the likelihood of infection. This variable represents at least part of the so-called 'species barrier effect,' whereby experimental transmission of infection is most efficient if the donor and recipient are of the same species and PrP genotype.

The magnitude of the species barrier between cattle and pigs, poultry and fish is not known, but some indication of this may be obtained from consideration of the degree of PrP amino acid sequence homology between species. There is between 87.3% and 91.5% homology between cattle and pig PrP, comparing nucleotide and amino acid sequences respectively (37). While homology between species of bird is approximately 90%, the identity between birds and mammals is very low, at approximately 30% (52). Research on the PrP gene of fish is less advanced. Gibbs Jr and Bolis (22) reported detection of a normal isoform of amyloid protein in spawning salmon and Suzuki *et al.* (46) report the detection of a possible homologue to the PrP gene in pufferfish (*Fugu rubripes*). This contrasts with the failure of Joly *et al.* (27) to identify sequence similarities to known prions by searching fish databases. Appropriate comparisons with mammalian sequences will be necessary, but evolutionary divergence can be expected to result in a significant lack of homology.

The carrier state

Although studies initiated in the early years of the BSE epidemic anticipated a search for overt clinical disease and distinct pathology in challenged animals, subsequent research in laboratory rodents has raised concerns about the possible existence of a carrier state. A number of studies in rodents (24, 41, 48) have shown that the absence of clinical disease during the natural life span of the recipient species does not necessarily prove that transmission was unsuccessful. Evidence of replication within the new host, that does not result in clinical

disease, death and recognised pathological change, prompts debate on the existence of unrecognised infected animals in exposed populations. While the debate may focus on whether such animals represent true carriers that would never progress to clinical disease, or preclinically infected animals that will be killed for commercial reasons before reaching clinical onset, the key issue is that such animals could represent a hidden risk to others. By consumption of their tissues as food, or after processing as animal feed, further silent cycles of exposure could be initiated before such a state was recognised. This is essentially what occurred with BSE before 1986.

The development of more sophisticated tools for the detection of abnormal prion in tissues has enabled the identification of infectivity, or disease specific PrP as a surrogate marker, in sub-clinically infected animals. Although the absence of a sub-clinical carrier state cannot be absolutely ruled out in the experiments in pigs and poultry reported above, infectivity levels can be expected to be extremely low given the failure of multiple tools to detect evidence of transmission. Indeed, failure to detect infectivity on sub-passage between poultry provides greater reassurance. Nevertheless, the sensitivities of newer detection methods, such as transgenic mice expressing bovine or porcine PrP, and immunodiagnostics, present opportunities for further studies.

This is particularly important in eliminating the species barrier between inoculated experimental animals and the assay model. Wells *et al.* (50) estimated that the size of the cattle-pig species barrier reduced the effective oral exposure of pigs with BSE by as much as 100-fold. Additionally, despite the effectiveness of the mouse strains used for bioassay to detect infectivity in bovines, the pig-mouse species barrier possibly rendered the mouse assay too insensitive to detect infectivity in pigs. Sub-passage into a transgenic model expressing porcine PrP, or indeed into more pigs, may have been more sensitive, but would not guarantee a positive result. While the desire to prove the absence of the agent, or of infectivity, is understandable, proving negativity is inevitably impossible if infectivity is actually absent. The most that can be achieved is to prove that infectivity levels are extremely low. Given the lack of evidence of susceptibility of pigs to oral exposure with BSE and conflicting priorities for research, sub-passage into pigs has not yet been established. The situation will however be kept under review as alternative, sensitive tools become available.

A separate, but real, concern in countries where feed controls are restricted to feed manufactured for consumption by ruminants, is that pigs and poultry may also act as transient or passive carriers without actually becoming infected themselves. As a result, these animals could perpetuate cycles of transmission through feed and thereby undermine the effectiveness of feed bans. For example, where ruminant protein may still be fed to pigs and poultry, their offal may still represent a risk of recycling infectivity to ruminants if intestinal contents are still present at the time of rendering. In other words, if ruminant protein may be fed to pigs and porcine MBM may still be fed to ruminants, the intestine of the pig at slaughter, and consequently the porcine MBM, may contain ruminant protein. Similar arguments apply to avian intestinal contents.

Within-species recycling

A related area of concern, which has prompted occasional calls for further changes in feeding practices, is that of within-species recycling. This subject cannot adequately be reviewed in the context of this paper, but cannot be ignored either.

The BSE epidemic in Great Britain was undoubtedly propagated by the recycling of contaminated bovine protein. In other words, whether the origin of BSE was a spontaneous occurrence in bovines or the transmission of ovine scrapie to cattle, the subsequent recycling of infectivity in the absence of a species barrier presented minimal resistance to infection. As a consequence, it has been argued that within-species recycling of protein in other species would present similar dangers. Such a scenario could arise if species, such as pigs and poultry, became infected with a 'foreign' prion that had crossed the species barrier. Alternatively, the occurrence of a spontaneous prion infection in a species could be propagated more easily without having to cross a species barrier.

The risk of the former scenario is real, but as discussed in this paper, appears not to be likely, at least in the context of transmission of BSE or scrapie to pigs or poultry. The second scenario is theoretical, based upon a combination of factors, including the failure to identify external sources of infection as the origin of sporadic CJD. Given the absence of any historical evidence for the occurrence of spontaneous prion diseases in domestic species of livestock, implementing global prohibitions on the use of animal protein for animal feed, other than where the dangers have been proved, namely to ruminants, would seem premature. The massive economic and indeed environmental benefits of such recycling must be weighed against the theoretical risks, especially where alternative protein sources are scarce. Furthermore, it should not be forgotten that both pigs and poultry are omnivores that readily cannibalise their own species in certain circumstances. Thus the recycling of protein is less 'unnatural' than in ruminants. Any such changes should therefore be preceded by detailed and robust risk assessments.

Discussion

There can be no doubt that pigs and poultry in the UK, and probably in other countries, have been exposed to the agents of BSE and scrapie over many years. The distinct clinical signs observed in pigs challenged parenterally with BSE provide a basis on which a possible naturally occurring TSE might be recognised in the species. The essentially neurological nature of the presenting signs suggests that such animals are likely to be

reported as potential cases of notifiable diseases, especially classical swine fever (hog cholera), and subjected to close examination. This is dependent on sufficient numbers of pigs reaching maturity and remaining alive long enough to anticipate the onset of recognisable clinical signs, assuming feedborne infection early in life and an incubation period similar to BSE in cattle. Estimates indicate that sufficient numbers of breeding pigs survive to five or six years of age in the UK for there to have been over 1,000 such cases, had pigs been as susceptible as cattle to BSE by the oral route and with an equivalent incubation period (50). The absence of such cases, coupled with the apparent resistance of pigs to experimental oral challenge, suggest that the pig population in the UK was not historically infected. The absence of infectivity in the intestine and lymphoid tissue of orally challenged pigs, despite being exposed to doses much greater than would have been experienced naturally, also suggests that the likelihood of recycling infectivity in the sub-clinical state in the national herd was also improbable (50).

In the domestic fowl, the absence of clinical signs, or pathological changes, after parenteral challenges with BSE-infected cattle brain, supports the conclusions that the species barrier between cattle and poultry is too great to enable infection. However, sufficient numbers of domestic chickens in the UK population are unlikely to have survived to adult life to allow any opportunity for the observation of a naturally occurring TSE in the species.

A single published report of natural infection with a prion disease in domestic fowl (40) remains unconfirmed. The report refers to a single affected chicken, but brain sections reported to be from two affected birds were submitted by the author and examined by scientists at the Veterinary Laboratories Agency in consultation with other experts in the field. There was evidence of viral encephalitis, but no pathology suggestive of a TSE was present (11). The PrP immunostaining reported was considered to be non-specific. Further material from affected birds was not provided, and the name of the farm of origin was not divulged to the authorities, so thorough investigation of the incident was impossible. The report is therefore unacceptable as evidence of a naturally occurring TSE in domestic chickens.

A report in 1991 of possible spongiform encephalopathy associated with consumption of meat products in three ostriches in zoological collections in Germany between 1986 and 1989 remains unresolved (44, 45). Clinical signs of ataxia, inco-ordination of feeding behaviour, and difficulties in balancing, coupled with bilaterally symmetrical vacuolation of varying intensity in the brainstem and medulla were reported. Nevertheless, the manner of presentation made a systematic investigation difficult and toxic and nutritional causes were not ruled out. Transmissibility was not proven.

The situation in fish is more complex and published information on the susceptibility of fish to mammalian TSE agents is lacking. The species barrier, as determined by PrP gene sequence homology, may be considerable. At least in the UK, the voluntary withdrawal of MBM from the rations of farmed fish, reinforced by the 1996 ban on the use of this feedstuff, will have significantly reduced risks that the current farmed fish population is infected. The preferential use of fishmeal derived from fish caught at sea reduces the risk of within-species recycling, and of amplification of infectivity.

Despite the lack of evidence that pigs and poultry are susceptible to BSE, these outlets for the use of mammalian MBM should not be retained without close monitoring. In the UK there has been deliberate exclusion of specified risk materials from the diets of all livestock and pets, but the majority of feed controls are intended to prevent deliberate or accidental exposure of ruminants to ruminant protein. The continued use of ruminant protein in pig and poultry rations in some countries represents a real risk of cross-contamination of feed at the preparation stage. In addition, the legal inclusion of porcine MBM in ruminant feed may inadvertently perpetuate recycling of ruminant protein and BSE if intestinal contents are included. Thus, while pigs and poultry may not in themselves represent a risk to each other, to other animals or to consumers, they may represent obstacles to the effective prevention of continued exposure of ruminants.

Acknowledgements

The authors wish to acknowledge the help of P. Gallimore, N. MacDonald and R. Smullen for the information on fish feeding and to thank H. Raine and P. Sanderson for critical comment on text relating to diets for monogastric animals. G.A.H. Wells, S.A.C. Hawkins and S.J. Ryder kindly supplied pre-publication data and offered critical comment on the text. The experimental investigations were funded primarily by the Ministry of Agriculture, Fisheries and Food (now Department for Environment, Food and Rural Affairs [DEFRA]), although more recently, the projects were transferred to the Food Standards Agency research portfolio.

Possibilités d'encéphalopathies subaiguës spongiformes transmissibles chez les animaux de rente non ruminants et les poissons

D. Matthews & B.C. Cooke

Résumé
Les porcins et les volailles du Royaume-Uni ont incontestablement été exposés à l'agent de l'encéphalopathie spongiforme bovine (ESB). Ils ont ingéré les même protéines de ruminants qui ont été à l'origine de l'épizootie d'ESB dans le cheptel bovin. Or, aucun foyer de cette maladie n'a été rapporté chez ces espèces. Certaines expériences ont révélé que les porcs étaient sensibles à une inoculation parentérale répétée, mais qu'ils étaient résistants à une contamination par voie orale avec de l'encéphale de bovins atteints d'ESB. Des données récentes, encore incomplètes, donnent à penser qu'ils seraient également résistants à une contamination par voie orale avec l'agent de la tremblante du mouton. Des études réalisées sur des poulets domestiques ont montré que ces derniers étaient résistants à une contamination par voie parentérale et orale. Malheureusement, aucun résultat n'a été publié sur la sensibilité des poissons à l'infection. Les auteurs décrivent des découvertes fortuites dans l'encéphale de porcins non exposés qui, en d'autres circonstances, auraient soulevé l'inquiétude quant à l'existence d'une forme d'encéphalopathie subaiguë spongiforme transmissible dans la population porcine mondiale.

Mots-clés
Encéphalopathie subaiguë spongiforme transmissible – Farine de viande et d'os – Poisson – Porcin – Poulet – Prion – Protéine du prion – Volaille.

Posibilidades de que el ganado no rumiante o los peces contraigan encefalopatías espongiformes transmisibles

D. Matthews & B.C. Cooke

Resumen
En el Reino Unido hay sin la menor duda porcinos y aves de corral que se han visto expuestos al agente infeccioso de la encefalopatía espongiforme bovina (EEB). Aunque consumieron las mismas proteínas de rumiantes que provocaron la epizootia de EEB en el ganado vacuno, no existe indicio alguno de que en dichas especies se haya manifestado la enfermedad. Varias investigaciones experimentales han puesto de relieve que los porcinos son susceptibles a la infección por exposición parenteral múltiple pero resistentes al contagio por vía oral tras la ingestión de encéfalo de bovinos infectados. Aunque incompleta, la información disponible hoy en día lleva a pensar que los porcinos son también resistentes a la exposición oral al prurigo lumbar. Los estudios realizados con pollos domésticos indican que son resistentes al contagio por vía tanto parenteral como oral. Lamentablemente, hay datos no publicados que atestiguan la susceptibilidad de los peces a la infección. Los autores también describen algunas observaciones fortuitas en el encéfalo de porcinos no expuestos, que

podrían suscitar inquietud por la eventual presencia de una encefalopatía espongiforme transmisible en poblaciones porcinas de cualquier lugar del mundo.

Palabras clave
Ave de corral – Encefalopatía espongiforme transmisible – Harina de carne y huesos – Pez – Pollo – Porcino – Prión – Proteína del prión.

References

1. Anon. (1939). – The Defence General Regulations. Statutory Instrument No. 129. Her Majesty's Stationery Office (HMSO), London, 129 pp.
2. Anon. (1942). – The Feedingstuffs (Regulation of Manufacture) Order 1942. Statutory Instrument No. 2031. Her Majesty's Stationery Office (HMSO), London, 8 pp.
3. Anon. (1946). – The Feedingstuffs (Regulation of Manufacture) (Amendment No. 10) Order 1946. Statutory Instrument No. 2124, Her Majesty's Stationery Office (HMSO), London, 6 pp.
4. Anon. (1947). – The Feedingstuffs (Regulation of Manufacture) (Amendment No. 2) Order 1947. Statutory Instrument No. 2816, Her Majesty's Stationery Office (HMSO), London, 7 pp.
5. Anon. (1948). – The Feedingstuffs (Regulation of Manufacture) (Amendment) Order 1948. Statutory Instrument No. 2388. Her Majesty's Stationery Office (HMSO), London, 11 pp.
6. Anon. (1988). – The Bovine Spongiform Encephalopathy Order 1988. Statutory Instrument No. 1039. Her Majesty's Stationery Office (HMSO), London, 6 pp.
7. Anon. (1990). – The Bovine Spongiform Encephalopathy (No. 2) Amendment Order 1990. Statutory Instrument No. 1930. Her Majesty's Stationery Office (HMSO), London, 3 pp.
8. Anon. (1996). – The Bovine Spongiform Encephalopathy (Amendment) Order 1996. Statutory Instrument No. 962, Her Majesty's Stationery Office (HMSO), London, 2 pp.
9. Anon. (2001). – Feed Facts Quarterly. HGM Publications, HGM House, Nether End, Baslow, Bakewell, 17-18.
10. Bossers A., Belt P.B.G.M., Raymond G.J., Caughey B. & de Vries R. (1997). – Scrapie susceptibility-linked polymorphisms modulate in the *in vitro* conversion of sheep prion protein to protease-resistant forms. *Proc. natl Acad. Sci. USA*, **94**, 4931-4936.
11. Cawthorne R.J.G. (1997). – Failure to confirm a TSE (transmissible spongiform encephalopathy) in chickens. *Vet. Rec.*, **141**, 203.
12. Dawson M., Wells G.A.H., Parker B.N.J. & Scott A.C. (1990). – Primary parenteral transmission of bovine spongiform encephalopathy to the pig. *Vet. Rec.*, **127**, 338-339.
13. 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 (R. Bradley & B. Marchant, eds). Commission of the European Communities, Kluwer Academic Publishers, Dordrecht, 25-32.
14. 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, hamsters, pigs and domestic fowl. *In* Transmissible spongiform encephalopathies (R. Bradley & B. Marchant, eds). A consultation on BSE with the Scientific Veterinary Committee of the Commission of the European Communities, 14-15 September 1993, Brussels. Document VI/4131/94-EN. European Commission, Directorate-General for Agriculture, Brussels, 161-167.
15. European Commission (EC) (1994). – Commission Decision 94/381 of 27 June concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein. *Off. J. Eur. Communities*, **L 172**, 7 July, 23-24.
16. European Commission (EC) (2000). – Council Decision 2000/766/EC of 4 December concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein. *Off. J. Eur. Communities*, **L 306**, 7 December, 32-33.
17. European Commission (EC) (2001). – Council Regulation (EC) 999/2001 of the European Parliament and of the Council of 22 May, laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. *Off. J. Eur. Communities*, **L 147**, 31 May, 1-40.
18. European Commission (EC) (2001). – Commission Regulation (EC) 1326/2001 of 29 June, laying down transitional measures to permit the changeover to the Regulation of the European Parliament and of the Council (EC) No. 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies, and amending Annexes VII and XI to that Regulation. *Off. J. Eur. Communities*, **L 177**, 30 June, 60-67.
19. European Commission (EC) (2002). – Commission Regulation (EC) 270/2002 of 14 February amending Regulation (EC) No 999/2001 of the European Parliament

and of the Council as regards specified risk material and epidemio-surveillance for transmissible spongiform encephalopathies and amending Regulation (EC) No. 1326/2001 as regards animal feeding and the placing on the market of ovine and caprine animals and products thereof. *Off. J. Eur. Communities*, **L 045**, 15 February, 4-15.

20. Fraser H., McConnell I., Wells G.A.H. & Dawson M. (1988). – Transmission of bovine spongiform encephalopathy to mice. *Vet. Rec.*, **123**, 472.

21. 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.

22. Gibbs C.J. Jr & Bolis C.L. (1997). – Normal isoform of amyloid protein (PrP) in brains of spawning salmon. *Molec. Psychiatry*, **2**, 146-147.

23. 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* Proc. 15th International Pig Veterinary Society Congress, July 5-9, Birmingham. Nottingham University Press, Nottingham, 168.

24. Hill A.F., Joiner S., Linehan J., Desbruslais M., Lantos P.L. & Collinge J. (2000). – Species-barrier-independent prion replication in apparently resistant species. *Proc. natl Acad. Sci. USA*, **97,** 10248-10253.

25. Hunter N. (1997). – Molecular biology and genetics of scrapie in sheep. *In* The genetics of sheep (L. Piper & A. Ruvinsky, eds). CAB International, Wallingford, 225-240.

26. Hunter N., Cairns D., Foster J.D., Smith G., Goldmann W. & Donnelly K. (1997). – Is scrapie solely a genetic disease? *Nature*, **386**, 137.

27. Joly J.S., Nguyen V. & Bourrat F. (2001). – Conservation of the prion protein in vertebrates (Conservation des 'prions' chez les vertébrés). *Prod. anim. INRA*, **14**, 91-96.

28. Kellner O. (1915). – The scientific feeding of animals (translated by W. Goodwin). Duckworths, London, 221-222.

29. 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 (J. Bjornsson, R.I. Carp, A. Love & H.M. Wisniewski, eds). *Ann. N.Y. Acad. Sci.*, **724**, 210-220.

30. King J.O.L. (1978). – An introduction to animal husbandry. Blackwell Scientific Publications, Oxford, 357, 359 & 386.

31. Kocisko D.A., Priola S.A., Raymond G.J., Chesboro B., Lansbury P.T. & Caughey B. (1995). – Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proc. natl Acad. Sci. USA*, **92**, 3923-3927.

32. Kortz G.D., Meier W.A., Higgins R.J., French R.A., McKiernan B.C., Fatzer R. & Zachery J.F. (1997). – Neuronal vacuolation and spinocerebellar degeneration in young Rottweiler dogs. *Vet. Pathol.*, **34,** 296-302.

33. Ministry of Agriculture, Fisheries and Food (MAFF) (1924). – Report of the Fertilisers and Feedingstuffs Advisory Committee. Her Majesty's Stationery Office (HMSO), London.

34. Ministry of Agriculture, Fisheries and Food (MAFF) (1925). – Report of the Fertilisers and Feedingstuffs Advisory Committee, Her Majesty's Stationery Office (HMSO), London.

35. Ministry of Agriculture, Fisheries and Food (MAFF) (1926). – Fertilisers and Feedingstuffs Act 1926, 321. Her Majesty's Stationery Office (HMSO), London, 33 pp.

36. Ministry of Agriculture, Fisheries and Food (MAFF) (1932). – Fertilisers and Feedingstuffs Regulations 1932. Her Majesty's Stationery Office (HMSO), London, 39 pp.

37. Martin T., Hughes S., Hughes K. & Dawson M. (1995). – Direct sequencing of PCR amplified pig PrP genes. *Biochim. biophys. acta*, **1270**, 211-214.

38. McGill I.S. & Wells G.A.H. (1993). – Neuropathological findings in cattle with clinically suspect but histologically unconfirmed bovine spongiform encephalopathy. *J. comp. Pathol.*, **108,** 241-260.

39. Morrison W.B. (1928). – A handbook for the student and stockman. Cornell University, Ithaca, New York, 185-186.

40. Narang H. (1997). – Failure to confirm a TSE in chickens. *Vet. Rec.*, **141**, 255-256.

41. Race R. & Chesboro B. (1998). – Scrapie infectivity found in resistant species. *Nature*, **392**, 770.

42. Raymond G.J., Hope J.H., Kokisco D.A., Priola S.A., Raymond L.A., Bossers A., Ironside J., Will R.G., Chen S.G., Petersen R.B., Gambetti P., Rubenstein R., Smits M.A., Lansbury P.T & Caughey B. (1997). – Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature*, **388**, 285-288.

43. 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.

44. Schoon H.A., Brunckhorst D. & Pohlenz J. (1991). – Beitrag zur neuropathologie beim Rothalsstrauss (*Struthio camelus*) – Spongiforme Enzephalopathie. *Vehr. ber Erkrank. Zootiere*, **33**, 309-313.

45. Schoon H.A., Brunckhorst D. & Pohlenz J. (1991). – Spongiforme Enzephalopathie beim Rothalsstrauss (*Struthio camelus*). *Tierärztl. Praxis*, **19**, 263-265.

46. Suzuki H.A., Kurokawa T., Hashimoto H & Sugiyama M. (2002). – CDNA sequence and tissue expression of *Fugu rubripes* prion protein-like: a candidate for the teleost orthologue of tetrapod PrPs. *Biochem. biophys. Res. Commun.*, **294**, 912-917.

47. Taylor D.M. & Woodgate S.L. (2003). – Rendering practices and inactivation of transmissible spongiform encephalopathy agents. *In* Risk analysis of prion diseases in animals (C.I. Lasmézas & D.B. Adams, eds). *Rev. sci. tech. Off. int. Epiz.*, **22** (1), 297-310.

48. Thackray A.M., Klein M.A., Aguzzi A. & Bujdoso R. (2002). – Chronic subclinical prion disease induced by low-dose inoculum. *J. Virol.*, **76**, 2510-2517.

49. Wells G.A.H., Scott A.C., Johnson C.T., Gunning R.F., Hancock R.D., Jeffrey M.J., Dawson M. & Bradley R. (1987). – A novel progressive spongiform encephalopathy in cattle. *Vet. Rec.*, **121**, 419-420.

50. Wells G.A.H., Hawkins S.A.C., Austin A.R., Ryder S.J., Done S.H., Green R.B., Dexter I., Dawson M. & Kimberlin R.H. (2003). – Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. *J. gen. Virol.* (in press).

51. 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.

52. Wopfner F., Weidenhöfer G., Schneider R., von Brunn A., Gilch S., Schwarz T.F., Werner T. & Schätzl H.M. (1999). – Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein. *J. molec. Biol.*, **289**, 1163-1178.

53. Zlotnik I. & Rennie J.C. (1958). – A comparative study of the incidence of vacuolated neurons in the medulla from apparently healthy sheep of various breeds. *J. comp. Pathol.*, **68,** 411-415.