Bovine spongiform encephalopathy: history and diagnosis of a decreasing epidemic

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Summary

In November 1986, at the United Kingdom Central Veterinary Laboratory, the brains of two cows presenting neurological symptoms were examined by two neuropathologists who noted lesions similar to those typically found in scrapie-affected sheep brains, i.e. spongiform tissue changes. After having collected epidemiological data, some British researchers linked the possible cause of the disease to certain animal proteins present in bovine feed. Epidemiology played a key role in furthering our understanding of the dynamics and the patterns of the disease and informed the initial control measures implemented. The characteristics fitting the epidemiological data were: a shape typical of an extended common source; all sick animals were index cases, in other words, there was generally one single case per herd; geographically, the cases were scattered throughout the UK. In 1996, after the detection of thousands of cases in the UK and some even in countries other than the UK, the disease (henceforth named bovine spongiform encephalopathy, BSE) was demonstrated to be zoonotic and to be responsible for a human spongiform disease: variant Creutzfeldt-Jacob disease. A qualitative indicator of the likelihood of the presence of BSE cases in any country (Geographical BSE Risk assessment) was requested by the European Parliament to the European Commission, and a general model to account for the modality of the spread of BSE in the cattle population and the role of the main risk factors was designed. The introduction of a comprehensive active surveillance plan in 2001 marked a turning point in the capability to accurately describe the geographical distribution of BSE and the trend of the epidemic across Europe. Within a few months reports of the disease unexpectedly arrived from everywhere in Europe. Since 2001, the trend of prevalence shows a strong and continuous decline across Europe, demonstrating the efficacy of all the measures put in place.

Keywords: atypical BSE, BSE, GBR, histopathology, surveillance

Introduction

In November 1986, an unusual laboratory finding triggered a series of events that would lead to wide-reaching changes in the meat-processing industry and bovine breeding practices, ultimately revolutionizing food safety legislation and consumer perception of health risks in the food chain. At the United Kingdom Central Veterinary Laboratory (CVL), the brains of two cows presenting neurological symptoms were examined by two neuropathologists (G.A. Wells and M. Jeffrey) who noted lesions similar to those typically found in scrapie-affected sheep brains (Wells *et al.*, 1987). The brains came from animals that had died over a year earlier

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in April 1985. Owing to its similarity to scrapie and peculiar histopathological features – spongiform tissue changes – the disease was classified as a prion disease and named bovine spongiform encephalopathy (BSE).

With time, more cases were identified and the threat of an epidemic of an unknown but serious disease loomed. One year after its first histopathological description, the clinical and pathological features of the disease became clearer. Evaluating the epidemiological data, figuring out the epidemic curve and thinking about the changes that had occurred in the feed production system, J.W. Wilesmith linked the possible cause of the disease to certain animal proteins present in bovine feed, known as meat-and-bone meal (MBM) (Wilesmith *et al.*, 1988). From the characteristics of the epidemic and the similarities observed between the new disease and scrapie in sheep, Wilesmith believed it likely that the cattle were being exposed to a scrapie-like agent via cattle feedstuffs containing ruminant protein. In July 1988, ruminant-derived proteins were banned from ruminant feeding in the UK: a sort of ban on cannibalism.

Wilesmith continued to collect information about the epidemic and to record its epidemiological features. On this basis he was able to pinpoint one (the most likely) of the problems in the system of processing animal carcasses in which their components (water, fat, grease) are separated to produce animal feed (rendering process) (Wilesmith *et al.*, 1988). Specifically, changes in the solvent extraction and the rendering temperature could have allowed the agent thought responsible for BSE to survive and spread into the bovine livestock. Summarizing, given that (1) the mean incubation period of BSE is about 5 years; (2) the disease was first observed in 1985; and (3) the traditional solvent extraction process (rendering) was largely discontinued between 1977 and 1984, it is likely that the emergence of the BSE epidemic was related to discontinuation of the use of solvent extraction by the rendering industry. The 'killer loop' is illustrated in Figure 1.

In the early stages of the BSE epidemic, epidemiology played a key role in furthering our understanding of the dynamics and the patterns of the disease and informed the initial
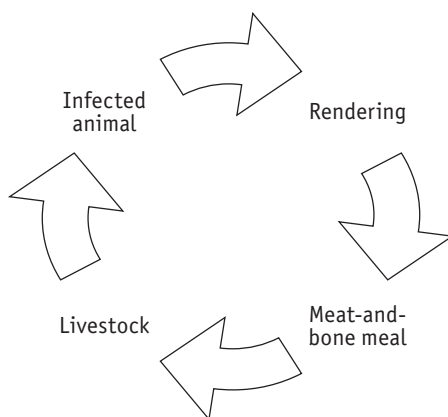

Figure 1. Cattle-to-cattle recycling of infection. Meat-and-bone meal may be the vehicle of infection.

control measures implemented. The characteristics fitting the epidemiological data were: a shape typical of an extended common source (Wilesmith *et al.*, 1988); all sick animals were index cases, in other words, there was generally one single case per herd; geographically, the cases were scattered throughout the UK, except for Scotland (here the rendering system procedures remained unchanged, as it was discovered later) where no cases were reported in the first period of the epidemic; dairy cows had a higher risk of BSE than beef cows, as was later demonstrated (Ducrot *et al.,* 2005; La Bonnardiere *et al.,* 2004; Ru G. *et al.,* 2007; Wilesmith *et al.,* 2000); the aetiological similarity between BSE and scrapie.

So far and in spite of all efforts, the origin of BSE is still unknown. It attracted major public concern in 1996 when evidence for the pathogenetic relationship emerged between BSE and a fatal neurodegenerative disorder in humans (now known as variant Creutzfeldt-Jakob disease; vCJD). With this link now established, BSE is considered a zoonotic disease potentially lethal in humans and acquired via contaminated food. Since then, the UK has had to deal with two epidemics, bovine and human, respectively.

Evidence for a causal link between the two diseases arose from biological and molecular strain-typing studies which clearly demonstrated that the BSE agent and the vCJD agent are one and the same, or at least have a similar pathogenicity in humans (Bruce *et al.*, 1997; Collinge *et al.*, 1996; Hill *et al.*, 1997; Scott *et al.*, 1999).

Again, epidemiology helped to reveal the association between BSE and vCJD. Their epidemic curves, the presumed response to various control measures, the time-scales of the two diseases, and their geographical occurrence all support this hypothesis, as do experimental studies.

In the decade between the detection of the first case in 1986 and the demonstration of BSE as a zoonotic disease in 1996, the British BSE epidemic curve showed a downward slope and consisted of about 169,000 cases (World Organisation for Animal Health, 2010). But in the meanwhile, trade in livestock and animal by-products dropped sharply and the frequent and intriguing question arose as to whether BSE could be present in countries other than the UK The answer was soon forthcoming: reports of BSE began to appear throughout Europe.

The impact of the BSE crisis was so strong that the European Parliament requested the European Commission (EC) to review its advisory system for public and animal health issues, particularly those related to agricultural production and food. The EU´s Scientific Steering Committee (SSC) was appointed to oversee eight specialized scientific committees dealing with subjects concerning food safety and public and animal health (Salman *et al.*, 2012). The SSC appointed a working group on transmissible spongiform encephalopathies (TSE) to assess the risk that a country could have undetected BSE cases within its own bovine population. The outcome was the geographical BSE risk assessment (GBR).

2. Geographical BSE risk assessment

By definition, GBR is a qualitative indicator of the likelihood of the presence of at least one animal within an autochthonous bovine population being infected with the BSE agent. In January 1998, the SSC defined the criteria for assessing country-specific risk factors.

In its 'Opinion on a method to assess the Geographical BSE-Risk (GBR) of Countries or Regions' (18 February 1999), the SSC proposed a general model designed to account for the modality of the spread of BSE in the cattle population in a country and the role of the main risk factors involved. The model represents a pattern with a positive feedback mechanism that tends, unless counteracted, to amplify the infection within the cattle population involved until a phenomenon of epidemic proportions appears. The course of events may be summarized as follows: BSE-infected material (slaughter waste, dead animals, etc.) enters the system, is treated in casting plants for the production of fats (rendering) and causes the contamination of MBM; cattle fed with the infected MBM are exposed to the infective agent; hence, the greater the number of animals exposed, the greater the number of animals infected; the greater the number of infected animals, the greater the number of infected animals (apparently healthy but actually in the incubation phase of the disease) regularly slaughtered and whose viscera are intended for rendering. This will eventually result in the contamination of increasing amounts of MBM and the persistence of the cycle.
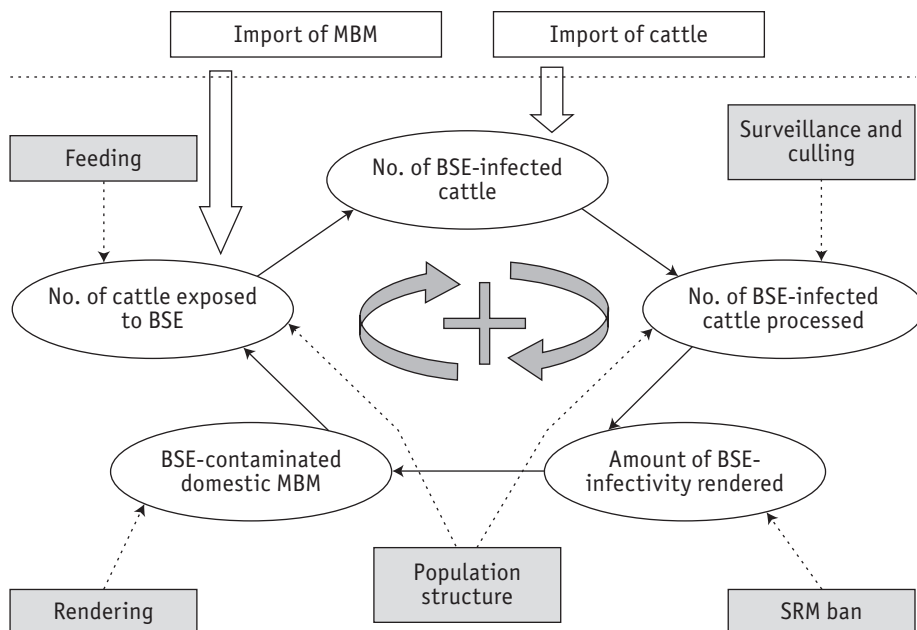

Figure 2. Simplified model of the assumed bovine spongiform encephalopathy (BSE)/cattle system, including factors responsible for the outbreak or amplification of the infection (Scientific Steering Committee, 1998). MBM = meat-and-bone meal; SRM = specified risk material.

The BSE agent is introduced into a country through imports of contaminated feedstuffs and/or infected cattle into the cattle husbandry system of the importing country. The husbandry system is defined as the interaction of a group of factors which determines whether or not the BSE agent will be recycled and amplified (Salman *et al.*, 2012).

The model is a simplification of what happens in reality. As such, it deliberately neglects the mechanism of vertical transmission and alternative, unidentified but still possible diffusion pathways (e.g. horizontal transmission). The simplified model, as used by the SSC, is shown in Figure 2.

Under the previous assumptions, the triggering point is the initial introduction of the BSE agent into the system from outside the domestic BSE/cattle system, and introduction is defined as an 'external challenge'. For all countries other than the UK, the import of contaminated feed or infected animals was the only possible initial source of BSE, since other putative sources were not included in the model because not scientifically confirmed. The ability of the national system to cope with this threat is called 'stability'. A stable system will not propagate or amplify an infection once introduced, whereas an unstable system will. Another step in the overall evaluation is to assess the 'interaction' between challenge and system stability, taking into account changes over time and the absence of precise information.

As mentioned above, potential domestic sources of BSE, independent of any external challenge, were not considered in the GBR assessment method.

The purpose of the assessment is to assign a country a specific risk level (i.e. a score) for the presence of the BSE agent. Four risk levels are currently distinguished:
1. highly unlikely;
2. unlikely but not excluded;
3. likely but not confirmed or confirmed at a lower level;
4. confirmed at a higher level.

On the basis of epidemiological studies performed to date (Wilesmith *et al.*, 1988, 1991, 1992a,b), several risk factors have been identified which can control, to a greater or lesser extent, the spread of infection as described by the SSC model. In particular, two centrally important factors – because they can interrupt the cycle (especially if combined) – are: (1) the effectiveness of treatments applied in the rendering process, which can reduce and, at least theoretically, eliminate the contamination of the MBM produced; this is obtained by maintaining a rendering temperature of 133 °C at 3 bar for periods of at least 20 consecutive minutes (as required by Directive 90/667/EEC; European Communities, 1990); and (2) exclusion of MBM from feeding the ruminants.

Other factors, though unable to halt the cycle, with a potentially significant effect on the increase or decrease of the number of cases of infection of the bovine population include:
• Import of foreign infected animals and/or contaminated meal can increase the number of infected animals in a country.

- Availability of tests for early diagnosis would allow the identification of carriers of infection and their consequent exclusion from processing.
- Exclusion from the rendering of specified risk material (SRM; mainly tissues from the central nervous system where nearly all of infectivity is confined) may substantially reduce the level of infectivity of the raw materials processed.
- Use of feed production processes to prevent the risk of cross-contamination (separation of production lines, storage facilities, and transport of MBM-free foodstuffs from those for pets).

On the basis of the afore-mentioned considerations, the SSC has identified eight major risk factors to take into account when assessing the risk characteristics of a certain cattle population. These factors may either stop or amplify the cycle of the infection:

1. Cattle population dynamics and structure affect the number of potentially exposed animals, and of those who become infected as well as those who, despite infection, are routinely slaughtered. A cattle population in which dairy cows are predominant and farms using large quantities of feed compounds will have a higher risk of exposure to the BSE agent than populations consisting mainly of beef cows raised on pasture. Moreover, given the growing body of evidence for infection during the incubation period, the age at slaughter will affect the infectivity titre of the material for the rendering.
2. Animal trade impacts on the number of infected animals who enter the cycle of the spread of the disease.
3. Animal feeding (production, use, import/export of MBM): the risk resides in potential unintentional cross-contamination.
4. The feed ban and related controls: controls are necessary to ensure that the ban is effectively enforced.
5. Legislation and controls on SRM: completely eliminating SRM from the food/feed chain does not necessarily ensure the absence of infectivity in the processed material.
6. Surveillance would be able to exclude a certain number of infected animals and clinically sick from the later stages of the cycle.
7. Efficiency of the rendering processes and the production of MBM: because an inefficient rendering system favours rapid spread of the disease, all efforts must be directed at preventing the introduction of animals or contaminated raw materials.
8. Strategies of culling the herd after the detection of an index case: animals which have shared the same exposure as the index case are to be culled as well. From this stems the importance of having an effective identification system of individual animals to ensure livestock traceability.

In 2006, the European Food Safety Authority (EFSA) was charged to carry out GBR. In 2007, after evaluating the SSC method, the EFSA revised the GBR methodology and suggested amendments and improvements. The main changes in the EFSA GBR with respect to the SSC GBR fall under three main categories: (1) changes in the external challenge assessment; (2) changes in the stability assessment; and (3) changes in the categories of assessment. Furthermore, the EFSA GBR addresses the possibility of assessing zones as defined in the World Organisation for Animal Health (OIE) terrestrial animal health code.

The revised challenge assessment in the EFSA GBR methodology introduced an adjustment for the size of the challenged cattle population; defined in more detail the steps for the assessment (i.e. acquisition of import data, determination of whether the imports entered the BSE/cattle system, estimation of the infectivity level in the imported material); clarified the rules for the inclusion or exclusion of imported material or animals; and introduced a weighting factor for the scaling of these imports.

The changes in the stability assessment entail the utilization of a semi-quantitative approach, which replaced the previous mostly qualitative approach to assess the impact of practices related to BSE infection (SRM utilization, rendering system and feeding system). Also, the EFSA GBR no longer categorizes countries. Instead, it assesses the overall challenge and the number of expected BSE cases and infections over time in a given country, with an estimation of the future course of the infection (EFSA, 2007).

3. Risk factors

In the first stages of the epidemic, the lack of an accurate surveillance system resulted in a biased assessment of the risk factors. A more reliable analysis has subsequently been carried out in the majority of European countries, thanks to the enforced active surveillance system put in place in 2001 (see below).

The main risk factors may be summarised as follows:
- The more a compound feed is used, the higher the risk of infection; the risk is higher for very large dairy farms because of the greater use of such feeds.
- Some studies have demonstrated a higher incidence of the disease in autumn-born cattle as compared to spring-born ones (Hoinville *et al.,* 1995; Wells *et al.,* 1987*;* Wilesmith *et al*., 1992a). This is likely due to seasonal differences in the use of concentrates: stable feeding during autumn and winter and grazing on pasture during spring and summer.
- La Bonnardière *et al.* (2004) investigated the relationship between milk yield and the risk of BSE; however, milk yield is only an approximation of the amount of concentrates given to cattle.
- Production type and, consequently, herd size play a significant role in the spread of infection, though not consistently across countries (Donnelly *et al.,* 1997; Ducrot *et al.,* 2008; Griffin *et al,* 1997; Ru *et al.,* 2007).
- In general, dairy cattle have a higher risk than beef suckler herds (Ru *et al.,* 2007; Wilesmith *et al*., 1992a).
- Stevenson *et al.* (2005) investigated two birth cohorts (those born before the July 1988 ban on feeding ruminant-derived MBM to ruminants and those born after that date) to determine area-level risk factors for BSE. The authors concluded by stating that spatially localised influences were operating in divergent ways during the two phases of the epidemic. For the cohort of cattle born after the July 1988 ban on feeding MBM went into effect, the area-level BSE risk was additionally associated with greater numbers of pigs per area relative to cattle. These findings support the influential role of low-level cross-contamination of cattle feed by pig feed in BSE incidence as the epidemic evolved.

4. BSE epidemiological surveillance

Until 1990, no cases of BSE had occurred outside the UK and Ireland. When the first cases were identified in Switzerland and Portugal, it became immediately clear that surveillance operations against BSE would prove particularly difficult. The main reasons lie in the relative rarity of the disease (which exceeded the annual incidence of 100 cases per million cattle in the UK alone, and most recently, in Portugal) and in the absence of applicable diagnostic tests on live animals. To complicate matters further, BSE has a very long incubation period (5 years). Despite the huge number of cases detected in the UK, it still seemed too early to think about a surveillance strategy based on systematic diagnostic tests on healthy, regularly slaughtered animals.

In 1992, the OIE published the first edition of a chapter of the international animal health code specifically devoted to BSE. The OIE recommended that the BSE status of a country should be determined according to three criteria: (1) the results of risk assessment; (2) the measures put in place to manage the BSE risk; and (3) the incidence rates reported by the surveillance plan. With regard to the last, the OIE suggested taking an intensive passive surveillance approach. Combined with the mandatory reporting of all suspected cases (with clear untreatable neurological signs), control activities should have been concentrating on categories of animals in which the probability of detection of BSE cases had to be higher, i.e. animals imported from infected countries, fed with contaminated MBM, and unsolved cases of death of unknown origin.

The 68th OIE general session (22-26 May 2000) approved a reviewed version of the BSE chapter which states that the presence of the disease can only be determined on the basis of the following criteria:
- Risk assessment which identifies all potential exposures: (1) MBM consumption; (2) importation of MBM; (3) importation of potentially infected animals or ova/embryos; (4) epidemiological situation of TSEs in a country; (5) level of knowledge of the livestock structure; and (6) source of animal waste, parameters of treatment and methods of production.
- Continuous awareness programs for veterinarians, farmers and any other professionals involved.
- Mandatory notification and diagnostic testing of all cattle showing clinical signs consistent with BSE.
- Continuous monitoring systems which take into account the risk factors listed and meet the criteria defined in a special appendix to the text.
- Diagnostic examination of the samples in an approved laboratory.

In 1998, sharing the OIE strategy, the European Union initiated a study to characterize the risk of the spread of BSE throughout the member states and the exporting third countries. This shared approach to epidemiological surveillance based solely on passive reporting of cases revealed its ineffectiveness, wrongly suggesting a limited spread of the disease outside the UK.

In January 1999, Switzerland responded by implementing a targeted surveillance program (active surveillance) for BSE, using a specific Western blot test (Doherr *et al.,* 1999), through which all emergency-slaughtered adult cows and all animals dead on farm (fallen stock) were sampled for BSE screening. To complete the program, a random sample of about 7,000/year regularly-slaughtered cows was tested as well. The newly enforced plan led to a remarkable rise in BSE prevalence. It has been calculated that approximately 50% of the cases detected in 1999 would have been missed with passive surveillance alone.

Faced with the relative ineffectiveness of passive surveillance systems (based on the reporting of clinical suspects), EC moved quickly. During 1999, the EC organized the validation of various rapid tests that detect the prion protein PrP^{sc} (infection marker, see diagnostic section) in CNS tissue samples.

In spring of 2000, France followed suit, with the testing of risk populations in several regions considered at higher risk of BSE, given the scarce results of passive surveillance (Calavas *et al.,* 2001). At around the same time, nine countries identified at least one BSE case in their naive cattle population (Belgium, Denmark, France, Germany, Ireland, the Netherlands, Portugal, Spain, and Switzerland) and all the cases (except for Switzerland) were identified by passive surveillance.

A major limitation of passive surveillance is that it relies on clinical signs of disease. In BSE, however, subclinical infections or preclinical stages of the disease go unrecognised by this tool, which is an otherwise very useful means for the farmer to report diseases early (Doherr *et al.,* 2001).

Based on the results of the enlarged surveillance in Switzerland and France, the EU introduced active screening in risk populations for BSE in January 2001 and expanded surveillance to all cattle for routine slaughter over 30 months of age (in countries including Italy, also animals >24 months of age) to be implemented by July 2001. Through the introduction of active surveillance, countries which had long claimed to have no BSE cases detected their first ones. Furthermore, the incidence per million cattle >24 months of age noticeably increased in countries which had already detected BSE cases before the start of active surveillance (EC, 2002).

Over the past decade, European legislation setting the minimum age of cattle at which rapid testing is to be performed reflects, in part, the dynamics of the BSE epidemic, with a progressive extension of the cut-off age:
- Before 2001: only passive surveillance.
- From 1 January 2001 (EC, 2000, 2001): mandatory testing in healthy slaughtered animals aged >30 months and in animals at risk >24 months of age (fallen stock, emergency slaughter and animals with clinical signs at the *ante mortem*).
- From 1 January 2009 (EC, 2008): all animals >48 months (for 17 member states).
- From 1 July 2011 (EC, 2009b): healthy slaughtered animals >72 months; animals at risk >48 months (for 25 member states).
- In 2013: it is expected a new change that it is likely to stop the tests on healthy slaughtered animals.

In 2005, EC published a paper, entitled 'The TSE roadmap', which outlined possible changes in BSE surveillance and measures to control the disease, as well as establishing future steps in BSE policy on the definition and removal of SRM, the feed ban, and animal age at testing. In its introduction it states, 'We have come to the stage that amendments of certain measures could be envisaged without endangering the health of the consumer or the policy of eradicating BSE, provided that the positive trend continues and scientific conditions are in place. Indeed, different indicators already suggest a favourable trend in the BSE epidemic and a clear improvement of the situation in recent years due to the risk reducing measures in place' (EC, 2005).

Epidemiological surveillance of BSE, as envisaged by the TSE roadmap, does not play a direct role in the prevention of risk to animal or human health; instead, it serves as a useful tool to monitor spatial and temporal trends and to evaluate the effectiveness of the measures put in place. The TSE roadmap goes on to emphasize that, in terms of public health protection, the surveillance system has 'helped to strengthen consumer confidence and has played a role in the communication strategy'. Importantly, effective measures to mitigate the risk of exposure to BSE, and thus protect the health of animals and humans, include the prohibition of processed animal proteins for animal feeding and the exclusion of SRM from the food chain.

In 2010, the roadmap was revised and updated with 'The TSE roadmap 2' (EC, 2010b), which underscored the aim to continue with the review of risk mitigation measures while assuring a high level of food safety. The revised roadmap called for a stepwise approach to European regulations and policies on BSE as supported by solid scientific evidence. In this respect, the scientific advice that EFSA provides will be crucial in informing future policy options. The new goal is now to monitor situations of a potential re-emergence of BSE or the emergence of a new TSE agent in the cattle population.

5. BSE in Europe

The introduction of a comprehensive active surveillance plan in 2001 marked a turning point in the capability to accurately describe the geographical distribution of BSE and the trend of the epidemic across Europe. Within a few months, the epidemiological BSE pattern (previously based on data only from passive surveillance) radically changed shape when reports of the disease unexpectedly arrived from countries other than the UK. Contrary to what was happening in Britain, where the number of BSE cases decreased by one third (from 2,301 to 1,443) between 2000 and 2001, the total number of cases doubled (from 515 to 1,012) in the rest of Europe and Japan also reported its first three cases. However, at least in Western Europe, 2001 also marked the beginning of the slow decline of BSE, demonstrating the efficacy of the control measures put in place since the mid 1990s: the MBM ban and the exclusion of SRM (potentially infected cattle tissues) from food and feed chains to mitigate the risk of exposure to infection.

Also, active surveillance subsequently allowed the identification of atypical forms, so-called L-BSE (BASE) and H-BSE, which are probably very rare, different diseases, with epidemiological features different from those of classical BSE.

In the years to follow, the trend of classical BSE and, thus, the effectiveness of enforcement, would be monitored through the testing of huge numbers of cattle (10 million cows per year on average in the EU). Now, some 12 years later, the problem seems to be solved, and since 2005 the EU has been setting up an exit strategy from the crisis (TSE roadmap; EC, 2005), providing for the gradual easing of the measures.

On the basis of the data collected from the information systems in place, and available from the annual reports EC published between 2002 and 2012, detailed information can be gleaned about the prevalence and incidence of BSE in Europe and the temporal and geographical distribution of the disease. While surveillance activities have only recently been initiated in Romania and Bulgaria, data for at least 17 countries have been available since 2001 (Austria, Belgium, Cyprus, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Portugal, United Kingdom, Slovenia, Spain and Sweden) and since 2004 for 8 other countries (Latvia, Lithuania, Malta, Poland, Czech Republic, Slovakia and Hungary). In addition, global data for BSE can be obtained at the website of the World Organization for Animal Health (World Organisation for Animal Health, 2010).

On the basis of provisional data from EC and OIE, the number of BSE cases identified worldwide in 2012 is small: 6 in Spain; 3 each in Ireland and Poland; 2 each in Portugal and the UK; 1 each in France, Switzerland, the United States, and Brazil (the diagnosis of this last case, however, was delayed for 2 years).

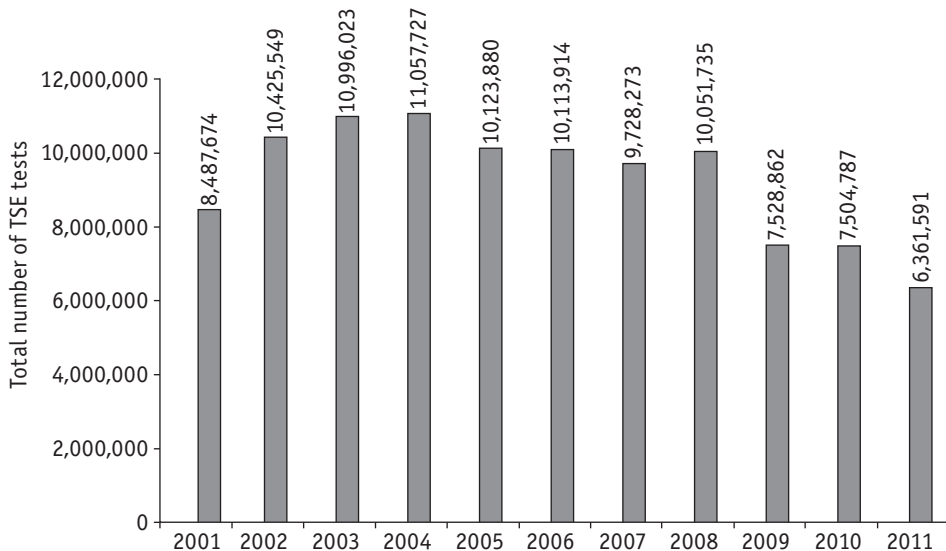The total number of tests performed in EU over 11 years (2001-2011) are shown in Figure 3 (source CE, 2012).

Figure 3. Total number of transmissible spongiform encephalopathies (TSE) tests performed in the period 2001-2011 in the 27 EU member states (EC, 2012).

The European figures up to 2011 show that the spread of the disease has been uneven across Europe. The frequency levels noted for the UK have always been higher than in the rest of Europe (even if active surveillance in the UK, at least initially, was applied to a sample base and cattle aged >30 months were excluded from testing). In the rest of Europe, the difference among countries is remarkably noticeable (Figure 4).

With regard to the temporal axis, the trend of prevalence shows a strong and continuous decline across Europe (27 Member States) where on the whole 7,852 cases have been identified (Figure 5).
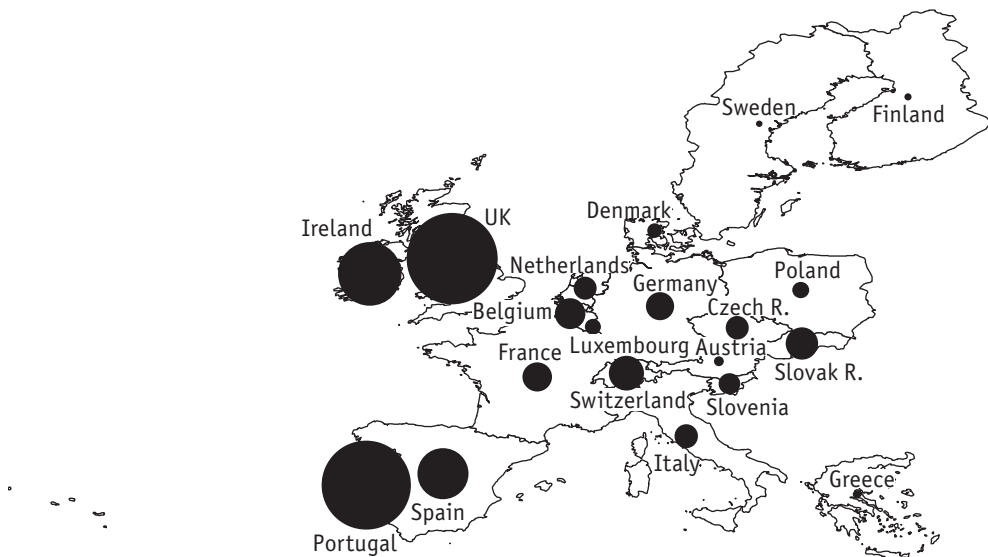
Figure 4. Average annual incidence by country (average annual cases per million cattle aged over 24 months) in the 2001-2011 period. The larger the dot, the higher the incidence (adapted from EFSA, 2012).
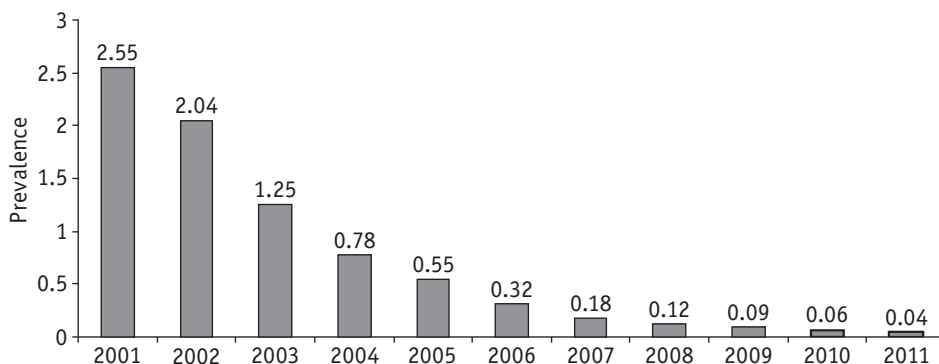

Figure 5. Prevalence (cases per 10,000 tests) by year between 2001 and 2011 (EC, 2012). Overall data from the 27 EU member states (7,852 cases recruited).

At the European level, assuming that the infection typically occurs in the first year of the calf's life, the decrease in the frequency of BSE was paralleled by an increase in average age of the cases, with a shift from 7 years in 2001 to 14.7 years in 2011 (Figure 6).

With the application of a range of effective measures at the European level, the presence of the disease decreased drastically. Furthermore, the increase in the average age of the cases indicates that the risk of exposure to infection, high in the mid 1990s, has gradually diminished and may eventually fade out. Such may not hold for Third Countries where reporting of BSE cases is sporadic, surveillance is relatively lax, and biosecurity measures to prevent BSE are not as stringent as those enforced in Europe.

Regarding Atypical BSE, the total number of cases officially recorded is 71 (source EFSA 2012), but not all the BSE cases have been characterized, as in the EU there will be a legal requirement for typing BSE positive cases only from July 2013. Epidemiological data reported by the EU MSs indicate that, over the last years, the number cases did not show any trend and that these cases were mainly identified in the fallen stock and healthy slaughtered animals older than eight years of age.
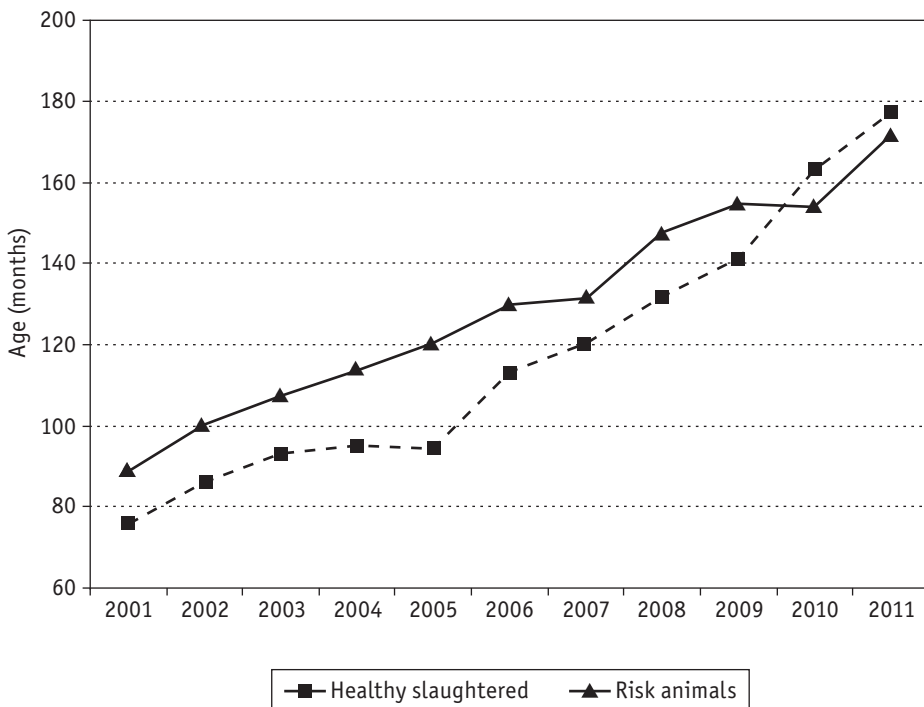

Figure 6. Average age (months) of bovine spongiform encephalopathy positive cases confirmed in the period 2001-2011 in the EU member states (EC, 2012). It can be taken as an indication that measures taken (mainly feed ban) have been effective.

6. Legislation

At present, Europe directly applies Regulation (EC) No 999/2001 (EC, 2001), as amended by more than 45 amendments establishing rules for the prevention, control, and eradication of certain TSEs. Under the provisions of the Regulation (EC) No 1069/2009 (EC, 2009a), which replaced Regulation (EC) No 1774/2002 (EC, 2002), new requirements for health and the collection and disposal of fallen stock and materials must be met.

7. Diagnosis

During the rapidly increasing epizootic in the late 1980s, BSE diagnosis was based on the histological identification of characteristic vacuolation in certain anatomical regions of the brain (Wells *et al.,* 1989). The detection of protein fibrils (equivalent to scrapie-associated fibrils; SAF) has been selectively used as an adjunct to routine diagnosis (OIE manual; World Organisation for Animal Health, 2010).

Since no method exists that can confirm the presence of BSE in live animals (World Organisation for Animal Health, 2010), cattle populations are monitored through passive and active surveillance programs. Under passive surveillance, cattle showing clinical symptoms consistent with BSE are tested for the disease. Active surveillance programs require BSE testing by rapid screening tests at the abattoir of apparently healthy cattle of a certain age and of fallen/dead stock.

Based initially on spongiform changes and examination of large case numbers, BSE diagnosis was established on the invariable predominance of brainstem lesions. Specifically, the medulla oblongata is the anatomic target site for diagnostic testing in both passive and active surveillance. In this context, the disease-associated (scrapie) conformer of the prion protein (PrPSc) is widely accepted as a consistent disease marker. With the exception of clinical examination and histopathology, all current diagnostic methods are based on the demonstration of this protein (World Organisation for Animal Health, 2010).

Samples collected in active transmissible spongiform encephalopathy (TSE) surveillance are screened with an approved rapid test, in accordance with Regulation (EC) No 999/2001 (EC, 2001) on the prevention, control and eradication of certain TSEs. In inconclusive or positive cases, the sample is submitted to confirmatory testing by histopathology, immunohistochemistry, immunoblotting, or demonstration of characteristic fibrils by electron microscopy.

7.1 Sampling

The preferred sampling site for testing is the brainstem at the level of the obex (the more aboral portion of the brainstem) because it is in the nuclei at this level that PrPSc accumulation begins in the central nervous system (CNS). Sampling is not recommended in cases in which the obex is absent, since samples from other brainstem portions will not show the early accumulation of PrPSc, thus precluding the certainty of a negative result. Obex

samples are obtained by inserting a long, spoon-shaped metal or disposable instrument with cutting edges through the foramen magnum (Gavier-Widen *et al.*, 2005) (Figures 7 and 8).
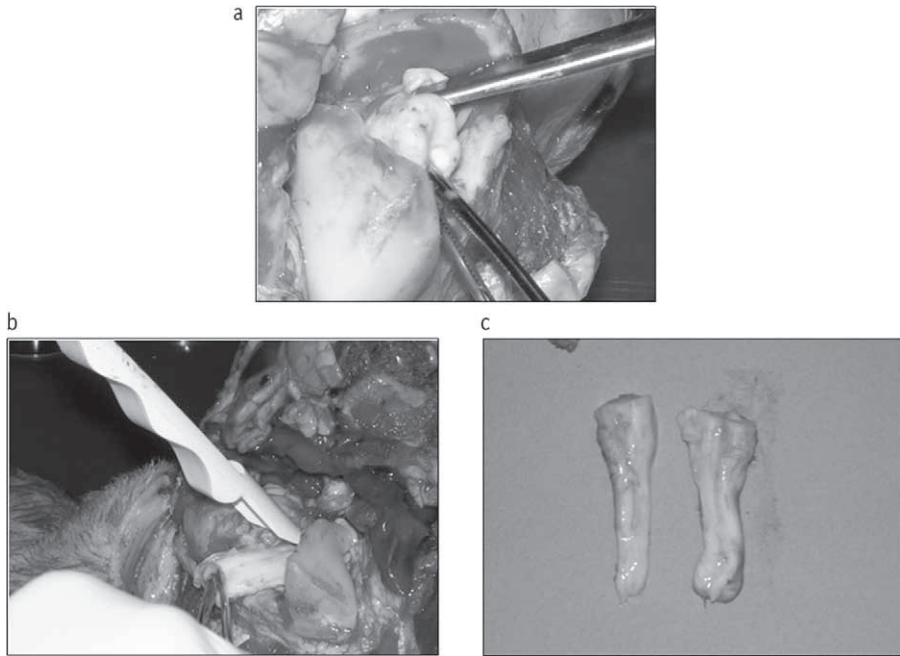

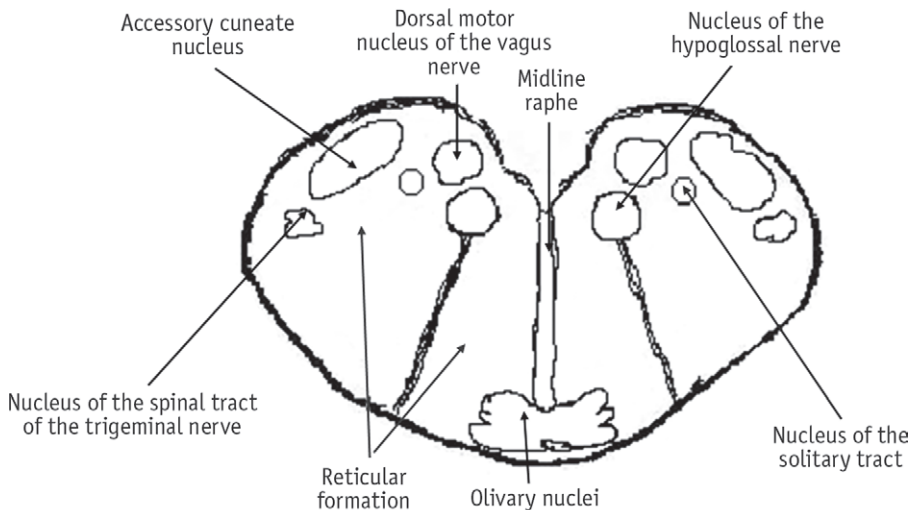*Figure 7. Withdrawal of the brainstem at the obex.*

Figure 8. Cross-section of the brainstem at the obex showing the target sites for bovine spongiform encephalopathy diagnosis by histopathology and immunohistochemistry.

7.2 Rapid tests

Regulation (EC) No 999/2001 (EC, 2001) requires that each member state develops an annual TSE monitoring programme that includes a rapid-test screening procedure. Rapid tests are approved by the Commission (see Annex X of EC, 2001) after being evaluated by the European Reference Laboratory (former CRL) under the scientific protocol validated by EFSA. The currently approved rapid tests (EC, 2010a) are:
- Prionics Check Western test;
- Enfer and Enfer TSE Kit version 2.0;
- Enfer and Enfer TSE Kit version 3.0;
- Bio-Rad TeSeE SAP rapid test;
- Prionics-Check LIA test;
- IDEXX HerdChek BSE-scrapie Antigen Test Kit, EIA;
- Prionics Check PrioSTRIP;
- Roboscreen Beta Prion BSE EIA Test Kit;
- Roche Applied Science Prion Screen.

These tests have high sensitivity and can be completed within 24 hours. They usually are completed within a few hours, making them suitable for high-throughput testing.

7.3 Confirmatory tests

All positive rapid tests results are confirmed using one of the confirmatory tests listed in the OIE manual (World Organisation for Animal Health, 2010). Confirmatory tests are performed by a recognised National Reference Laboratory or CRL. The tests have high specificity and serve to confirm clinical suspicion of disease or positive or inconclusive rapid test results. Recommended confirmatory test methods include histological examination, immunohistochemical (IHC) analysis, scrapie-associated fibril (SAF) detection and Western blot (WB) analysis (World Organisation for Animal Health, 2010).

After rapid testing, the brainstem sample is divided longitudinally along its midline: one part is fixed in 10% buffered formaldehyde solution for histological and IHC examination, and the other part is frozen at 20 °C for WB analysis or immunological detection of SAF proteins (Figure 9).

7.4 Histopathological examination

For histological examination, sections are stained with haematoxylin and eosin; an appropriate sample will include the solitary and the trigeminal tract nuclei. The characteristic histological BSE changes in the CNS are vacuolation of grey matter neuropil (spongiform change) and/or vacuolation of neurons, with a predilection for certain neuroanatomic locations (Wells *et al.*, 1989; Wells and Wilesmith, 1995); astrocytosis and neuronal degeneration may also be present (Simmons *et al.*, 1996).

Generally, the areas most severely affected are the solitary tract nucleus, the spinal tract nucleus of the trigeminal nerve, and the central gray matter of the midbrain (Figure 10).
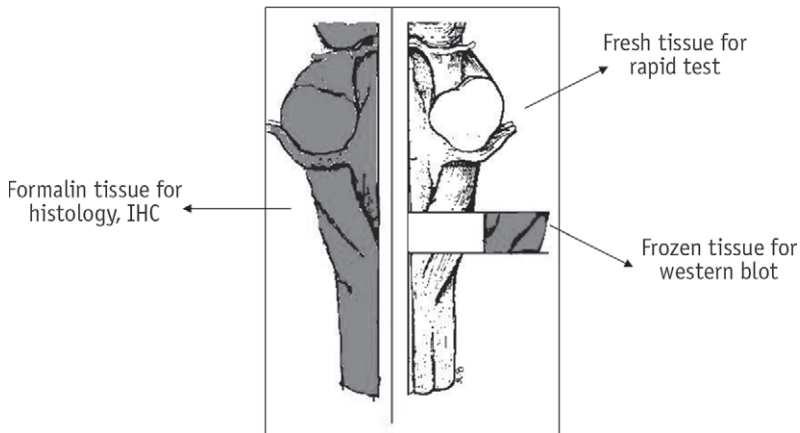
Figure 9. Sampling of the brainstem at the obex for confirmatory tests. IHC = immunohistochemical analysis.
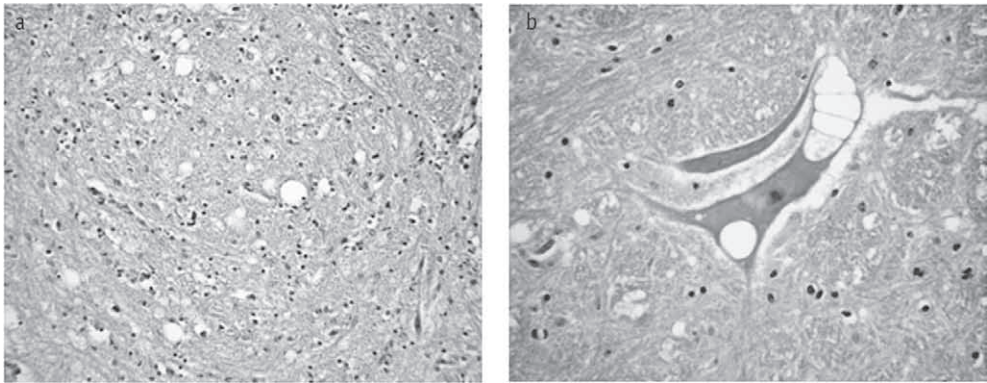

Figure 10. (a) Neuropil spongiosis (H&E; 20×). (b) Intraneuronal vacuolation (H&E; 40×).

7.5 Detection of scrapie-associated fibrils

The detection of purified scrapie-associated fibrils (SAF) is based on the use of negative-contrast electron microscopy (Stack *et al.,* 1996). Seldom used because of its low sensitivity, it may be an alternative method in autolysed samples or when only formalin tissue is available (Chaplin *et al*., 1998, 2002).

7.6 Immunohistochemical analysis

IHC analysis consistently reveals PrPSc accumulation in the brainstem at the obex, with a distribution similar to, but often more widespread than neuropil vacuolation. This technique uses specific antibodies after a specific pretreatment of sections to detect accumulated PrPSc *in situ*, and its sensitivity is comparable with immunochemical methods. In cattle, PrPSc cannot be readily detected in tissues outside the CNS, although limited involvement

of Peyer's patches in the distal portion of the ileum has been reported in experimentally induced and naturally acquired cases of BSE (Terry *et al.*, 2003).

7.7 Immunohistochemical PrPSc phenotype in the whole brain

Several morphologic types of PrPSc deposition can be found in the brain of BSE-affected cattle (Casalone *et al.*, 2006). The six principal types are (Figure 11):
- granular type characterized by granular PrPSc accumulations in the neuropil;
- glial type with PrPSc deposits branching out from the nucleus of a glial cell on its processes, conferring it a stellate appearance;
- coalescing type seemingly arising from the merging of granular PrPSc deposits to form amorphous or mesh-like masses;
- intraneuronal type with fine punctate PrPSc immunoreactivity throughout the neuronal cytoplasm;
- linear tract characterized by PrPSc deposits along neuronal processes;
- intraglial type with fine, punctate PrPSc immunoreactivity adjacent to the glial nuclei.

7.8 Western Blot

Western Blot analysis involves the detection of PrPSc in homogenates of unfixed CNS tissue. Various tests are currently available and more are under development. The test uses an antibody specific for PrP, whereby PrP and PrPSc are distinguished through an enzyme digestion step designed to remove PrP. Based on electrophoresis, Western blotting can distinguish the molecular weight and the glycosylation pattern of PrPSc (Figure 12).
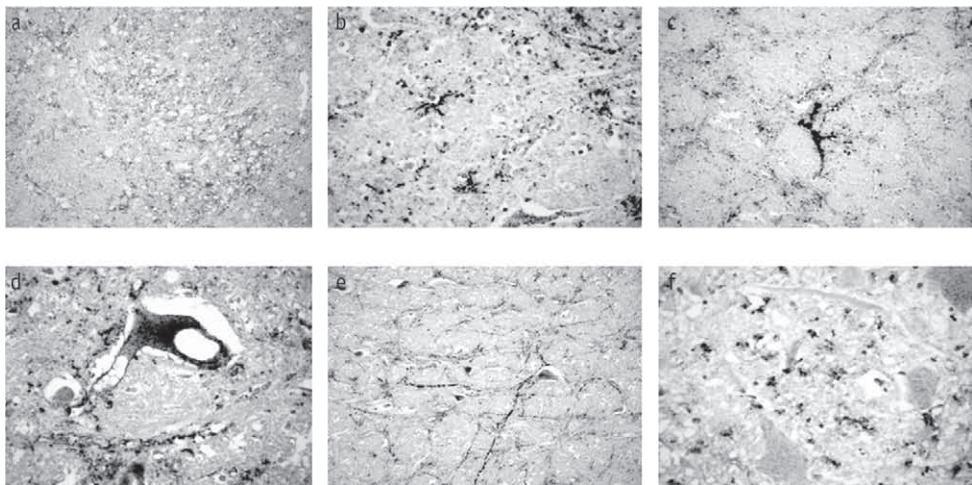

Figure 11. Patterns of prion protein PrPSc deposition (immunohistochemical analysis): (a) granular type (20×), (b) glial type (20×), (c) coalescing type (10×), (d) intraneuronal type (40×), (e) linear tract (10×), (f) intraglial type (40×).
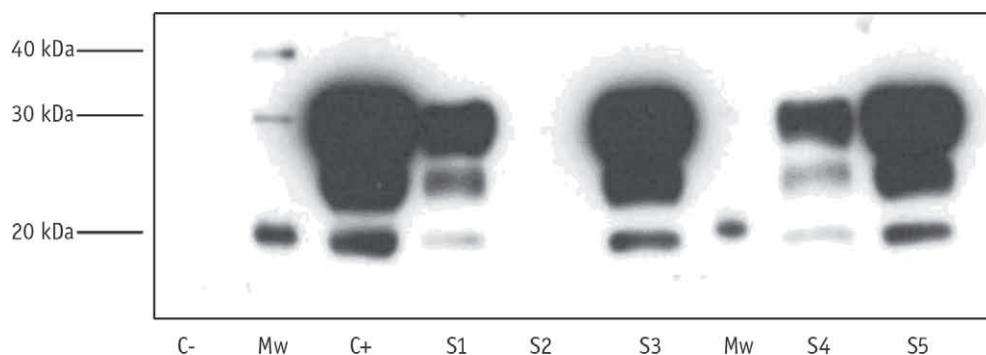
Figure 12. Confirmatory Western blot analysis of negative and positive bovine spongiform encephalopathy (BSE) cases. The membrane was probed with monoclonal antibody 6H4.C- and C+ = negative and positive BSE control samples, respectively; S1, S3, S4 and S5 = positive BSE cases; S2 = negative BSE case; Mw = molecular markers.

7.9 Differential diagnosis in cattle with neurological signs

The neurological signs were broadly classified as those suggestive of altered mental status or changes in sensation, and those that produced a change in animal posture or movement (Willesmith *et al.,* 1988). Changes in body weight or condition and milk yield were also recorded. The incubation period is roughly from 4 to 5 years in cattle.

Regulation (EC) No. 999/2001 (EC, 2001) requires that all clinically suspected BSE cases be submitted to confirmatory testing. If the brainstem tests negative for BSE, the OIE manual (World Organisation for Animal Health, 2010) recommends that, in countries with a low incidence of BSE, clinically suspect cases are subjected to a standard neuropathological approach in which the whole brain is sampled. The brain is divided and the larger portion is fixed in 10% buffered formaldehyde solution for histological examination and the smaller portion is frozen at 20 °C for microbiological analyses (Figure 13a). Brain sections from representative areas (medulla oblongata, pons, cerebellum, mesencephalon, diencephalon, and telencephalon) are stained with haematoxylin and eosin and examined to determine the presence or absence of lesions (Figure 13b).

Neuropathological examination of the entire brain from animals with nervous symptoms is a useful diagnostic practice when rapid tests result negative and in confirmed cases to establish whether or not the pathology is typical (Iulini *et al.,* 2012).

8. Atypical cases

Atypical cases of BSE have been reported from around the world and correctly identified by currently approved BSE rapid tests.
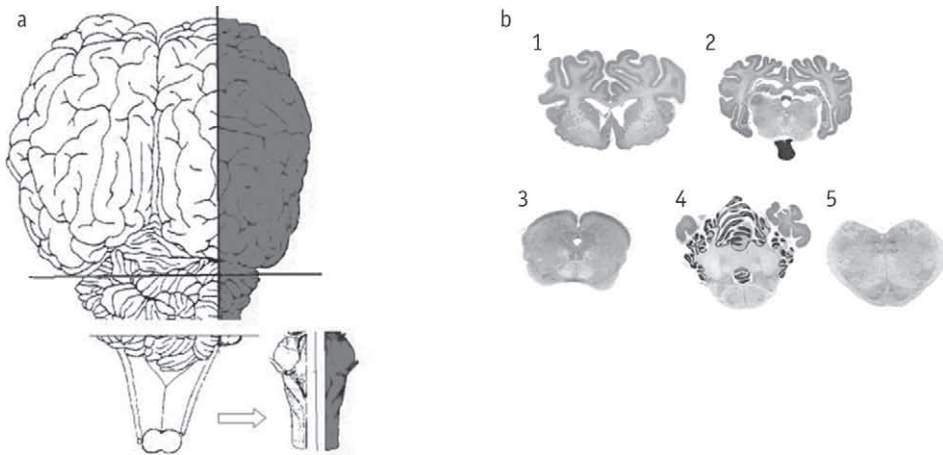
Figure 13. (a) Graphic representation of brain sampling. (b) Representative areas of the brain: (1) frontal cortex and basal ganglia; (2) thalamus and hippocampus; (3) midbrain; (4) pons and cerebellum; and (5) lower brainstem at obex.

The small number of variant forms of TSE in cattle, operationally defined as bovine amyloidotic spongiform encephalopathy (BASE) or classified as L-type and H-type BSE based on the mass of the unglycosylated PrP fragment on Western blot analysis (Casalone *et al.,* 2004; Jacobs *et al.,* 2007) have yielded little clinical information and have been identified mainly in older, apparently healthy or fallen stock cattle.

More detailed characterization of PrPSc deposition throughout the brain of BSE-affected cattle uncovered the first example of atypical L-type BSE in Italy in 2004 (Casalone *et al.,* 2004). Two older animals were found to be affected by a variant form of BSE, classified as L-type.

Western blot analysis revealed a dominant monoglycosylated PrPSc glycoform profile for these two atypical cases. Immunohistochemical analysis of brain sections revealed differences in the distribution and features of PrPSc immunoreactivity between C-type (classical) BSE and the Italian L-type BSE cases. Specifically, PrPSc in the L-type BSE cases was found to be more abundant in the forebrain than in the brainstem where PrPSc deposition was predominant in the C-type BSE cases. Unexpectedly, PrPSc deposition occurred in an unusual pattern referred to as amyloid plaques, (Porcario *et al.*, 2011) and the molecular signature resembled sporadic CJD seen in subjects with methionine/valine (M/V) substitution at PRNP codon 129 and type 2 PrPSc (M/V2) (Casalone *et al.*, 2004). On the basis of the unique neuropathological features of PrPSc amyloid plaques, the new L-type variant was named bovine amyloidotic spongiform encephalopathy (BASE). (Figure 14a and15).

Subsequent studies showed deposition of PrPSc within muscle fibers from cattle with natural and experimental BASE (Suardi *et al.*, 2012) (Figure 16). A study of intra-species transmission of a Japanese case of BASE showed that PrPSc was detectable by immunoblot first in the nerve roots and then in the peripheral nerves (Iwamaru *et al.*, 2010).
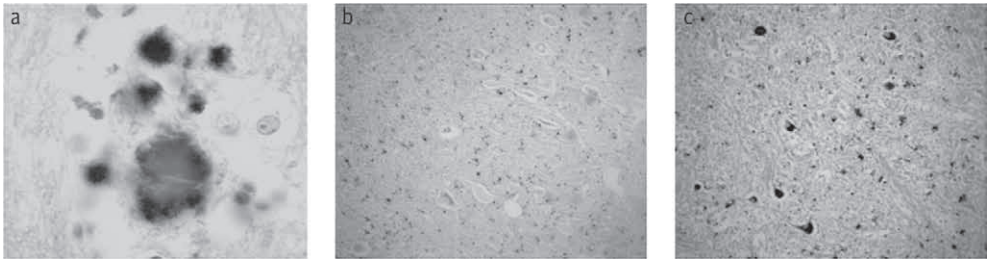
Figure 14. Patterns of prion protein PrPSc deposition (immunohistochemical analysis): (a) bovine amyloidotic spongiform encephalopathy: amyloid plaques (40×), (b) H-type: intraglial type (10×), (c) H-type: intraneuronal type (10×).
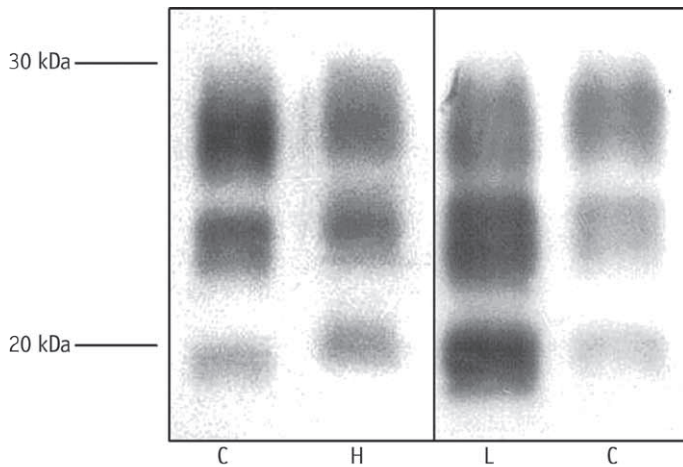

Figure 15. Western blot profiles of the prion protein PrPSc from C-, H- and L-type BSE isolates with monoclonal antibody 6H4.
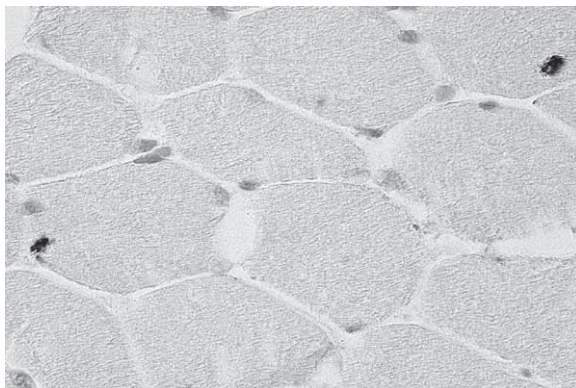

Figure 16. Prion protein PrPSc deposition in the peroneus muscles of cattle affected by natural bovine amyloidotic spongiform encephalopathy (immunohistochemical analysis; 40×).

The Italian case was discovered concomitantly with the detection of a second atypical BSE variant, the H-type, reported in France in 2004 (Biacabe *et al.*, 2004). H-type cases are characterized by a higher molecular weight of the unglycosylated fraction of PrPSc than C-type BSE (Figure 15) and by strong labelling with P4 (Harmeyer *et al.,* 1998) and 12B2 (Polak *et al.,* 2008) monoclonal antibodies. Both antibodies are directed to an N-terminal epitope that remains present after proteinase K (PK) cleavage of H-type BSE but not of C-type or L-type BSE-derived PrPSc.

Immunohistochemical studies on the brainstem from cases confirmed as H-type from Germany (Buschmann *et al.,* 2006) and the United States (Richt *et al.*, 2007; Richt and Hall, 2008) showed that the prevalent PrPSc deposition pattern was less intense, intraneuronal and intraglial, unlike that of C-type BSE, which is more intense, with mainly granular and linear tract deposition patterns (Figure 14b-c).

8.1 Characterization of atypical BSE

Both L-BSE and H-BSE agents are able to propagate experimentally in different animal species.

Experimental studies in cattle showed that BASE has a shorter incubation period than C-type and that the neuropathological PrPSc pattern reproduces those observed in field cases (Lombardi *et al.,* 2008). Instead, H-BSE has a longer incubation period than C-type (Balkema-Buschmann *et al.,* 2011).

The agent of L-BSE transmitted to wild-type mice and to transgenic mice expressing the VRQ allele of ovine PrP, and the agent of H-BSE inoculated into wild-type mice acquired a phenotype indistinguishable from the BSE agent (Baron *et al.*, 2011; Beringue *et al.*, 2007; Capobianco *et al.,* 2007). These findings suggest that BASE may converge into BSE through an intermediate host.

Recent studies have demonstrated the experimental transmission of BASE and H-BSE isolates to bovine-PrP transgenic mice. The transmissibility of the BASE strain was higher than either the BSE or H-type. Furthermore, the BASE-infected mice had a shorter incubation period than the BSE- or the H-type challenged mice. The three different BSE types presented as three distinct strains reproducing distinct disease phenotypes and biological properties (Buschmann *et al.*, 2006).

Intracerebral inoculation of brain from L-BSE-infected cattle to cynomolgus macaque induced a spongiform encephalopathy distinct in all its aspects (clinical, lesional and biochemical) from macaque BSE (Comoy *et al.,* 2008), and the incubation periods were shorter than for BSE. L-BSE was also transmissible to microcebes, with a shorter incubation than classical BSE (Baron *et al.*, 2008). Moreover, recent experiments demonstrated the transmissibility of L-BSE to macaque by the oral route (Comoy, 2010).

Intracerebral inoculation of BASE isolates produced TSE disease in two lines of mice overexpressing human PrP (Met129), exhibiting a molecular phenotype distinct from

classical BSE (Beringue *et al.*, 2008; Kong *et al.,* 2008). Both studies indicate that the BASE agent is transmissible to TgHu mice, whereas the H-type isolates failed to infect one line of 'humanized' mice (Beringue *et al.*, 2008).

More recently, results of experimental transmission of L-BSE and H-BSE to gene-targeted Tg mice expressing human PrP indicate that neither agent transmits infection and that these mice are highly resistant to infection with these animal TSEs (Wilson *et al.*, 2012).

Summarising:
- Transmission studies have shown that in some lines of mice both L-BSE and H-BSE display BSE-like characteristics and that the potential for interspecies transmission of atypical BSE is high.
- Experimental findings indicate that the L-BSE agent has the potential to be a zoonotic agent. In both primates and human PrP transgenic mice models, the virulence of the L-BSE agent is significantly higher than that of classical BSE.
- To date, H-BSE has not been reported as being transmissible to human PrP transgenic mice or to primates.

9. Conclusions

9.1 What has been achieved?

A constant decline in the BSE epidemic has been recorded throughout Europe. The different countries share the same risk factors and the measures put in place have demonstrated their efficacy. An overall relaxation of the control measures has been already set up and will be continued.

The great effort made by the European Union has been instrumental in promoting the almost complete disappearance of the disease from the EU.

The diagnostic tests are able to identify both the classical and the atypical forms of BSE.

The BSE epidemic has led to a turning point in the interpretation of food safety and in risk communication. Starting from the BSE crisis, the white paper first and the hygiene package later were born.

Furthermore BSE and all what is around is a virtuous example of the 'one health' concept, a strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans and animals. It is actually because of the complete integration of human medicine and veterinary medicine that TSEs, and more recently, atypical TSEs, have been so thoroughly studied in order to evaluate the implications for public health.

9.2 What has been neglected?

In terms of public health policy, it is hard to find a flaw in the 'system BSE', at least in Europe. Outside Europe little is known about the actual epidemiological situation, as a surveillance system similar to the European one has never been implemented.

9.3 What needs to be done?

The lack of an *in vivo* test is still a big gap in the diagnostic system, which hopefully will be filled by new ongoing research, to increase the efficacy of surveillance plans and reducing simultaneously the costs involved.

It is important to further investigate the real significance of atypical BSE and its potential role in causing a new 'BSE crisis' in case of a relaxation of control measures.

Similarly, it is important that a surveillance system is implemented in those non European countries where the epidemiological situation of BSE is still unknown, to avoid BSE to restart and to circulate in cattle populations.

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