

SCIENTIFIC OPINION

Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans¹

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ABSTRACT

The existing scientific evidence that links animal and human TSEs is reviewed and discussed. The challenges involved in identifying TSEs as zoonoses are described and the example of the process that led to the establishment of a link between Bovine Spongiform Encephalopathy (BSE) and variant Creutzfeldt-Jakob Disease (vCJD) is reviewed. The strain diversity of animal and human TSE agents and the factors influencing the capacity of TSE agents to cross the species transmission barrier are also discussed. The scientific opinion critically assesses the tools and methodologies currently available to study and evaluate the possible association of animal and human TSEs, focussing on epidemiological and laboratory methods. The available scientific evidence on Classical BSE, Atypical BSE (H-type and L-type), Classical scrapie, Atypical scrapie, Chronic Wasting Disease (CWD), Transmissible Mink Encephalopathy (TME) and human TSEs is reviewed. The conclusions state that, at present, the only TSE agent demonstrated to be zoonotic is the Classical BSE agent. Active screening has allowed the identification of three new forms of animal TSEs (H-type Atypical BSE, L-type Atypical BSE and Atypical scrapie), but the information obtained has major limitations due to the unknown sensitivity of the current monitoring system for these TSEs. There is no epidemiological evidence to suggest that Classical scrapie is zoonotic. The epidemiological data are too limited to conclude whether the Atypical scrapie agent has a zoonotic potential. Transmission experiments to human PrP transgenic mice or primates suggest that some TSE agents other than the Classical BSE agent in cattle (namely L-type Atypical BSE, Classical BSE in

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sheep, TME, CWD agents) might have zoonotic potential and indicate that that of the L-type Atypical BSE agent appears similar or even higher than that of the Classical BSE agent. A single study reported efficient transmission of a natural sheep Classical scrapie isolate to primates.

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KEY WORDS

Zoonosis, Transmissible Spongiform Encephalopathy, Bovine Spongiform Encephalopathy, Scrapie, Creutzfeldt-Jakob Disease

SUMMARY

Following to a request from the European Commission, the Panel on Biological Hazards (BIOHAZ) and the European Centre for Disease Prevention and Control (ECDC) were asked to deliver a scientific opinion on any possible epidemiological or molecular association between Transmissible Spongiform Encephalopathies (TSEs) in animals and humans. The opinion reviews and discusses the existing scientific evidence that links animal and human TSEs currently known.

The opinion first considers the definition of zoonoses and the principles for the identification of zoonotic diseases, which can be based on evidence gathered from both epidemiological and laboratory studies. The opinion describes the challenges involved in identifying TSEs as zoonoses, due to the specific characteristics of TSE infections/diseases, such as the nature of TSE agents, the occurrence of animal and human TSEs and the type of monitoring applied, the long incubation period of TSEs etc. The example of the process that led to establishing a link between Bovine Spongiform Encephalopathy (BSE) and variant Creutzfeldt-Jakob Disease (vCJD) is reviewed. The epidemiological and laboratory criteria that can be used to investigate such a link are described in detail, since those criteria might be useful for the identification of links between other animal and human TSEs.

The opinion discusses the strain diversity of the TSE agents described in sheep, goats, cattle, cervids and humans, based on the current knowledge, which highlights that multiple TSE agents exist in each species. The factors influencing the capacity of TSE agents to cross the species transmission barrier are then considered in detail, including the variability in host and donor PrP gene and protein, the TSE strain type involved and its interaction with the host PrP, and the route of infection.

The opinion critically assesses the tools and methodologies currently available to study and evaluate the possible association between animal and human TSEs. The use of epidemiology is discussed for TSEs in both animals and humans, and the possibility to compare the two sources of information is presented as a possible method to study the possible links.

Both *in vivo* and *in vitro* laboratory methods are considered and discussed, including neuropathology, transmission experiments involving different animal models (wild type and transgenic mice, primates and other species), biochemical methods, cell-free conversion assays, Protein Misfolding Cyclic Amplification (PMCA) and cell culture assays. Characteristics, advantages and disadvantages of the different methods are reviewed, including the opportunity to collate data from different types of experiments for the study of potential associations between animal and human TSEs.

The opinion then reviews the scientific evidence currently available for the different animal and human TSEs, including Classical BSE, Atypical BSE (H-type and L-type), Classical scrapie, Atypical scrapie, Chronic Wasting Disease (CWD), Transmissible Mink Encephalopathy (TME) and human TSEs. In particular, the following aspects are systematically discussed for each TSE agent: epidemiology, pathogenesis and *in vivo* and *in vitro* transmission experiments.

The opinion concludes that, at present, the only TSE agent demonstrated to be zoonotic is the Classical BSE agent. With regard to human TSEs, detected cases of sporadic CJD are randomly distributed in time and geographical location. These observations have been interpreted as a supportive argument that sporadic CJD is not environmentally acquired. However, the epidemiological evidence in relation to sporadic CJD cannot be regarded as definitive, and the possibility that a small proportion of cases are zoonotic cannot be excluded.

It also concludes that a series of uncertainties in relation to the epidemiological patterns of animal and human TSEs indicate that even a rough comparison of the present epidemiological patterns of human and animal TSEs other than Classical BSE is unlikely to be informative. Because of these

uncertainties, it is an imperative to continue to carry out systematic surveillance of human TSE diseases, and to continue and improve the surveillance of animal TSE diseases.

The opinion highlights that the active screening has allowed the identification of three new forms of animal TSEs (L-type Atypical BSE, H-type Atypical BSE and Atypical scrapie), but that the information obtained has major limitations due to the unknown sensitivity of the current monitoring system for these TSEs. There is no epidemiological evidence to suggest that Classical scrapie is zoonotic. The epidemiological data are too limited to conclude whether the Atypical scrapie agent has a zoonotic potential.

Transmission experiments to human PrP transgenic mice suggest that some TSE agents other than the Classical BSE agent in cattle (namely L-type Atypical BSE and Classical BSE in sheep agents) might have zoonotic potential, whereas for other agents there is no evidence provided of a zoonotic potential (H-type Atypical BSE and CWD), or no published studies are available (Classical and Atypical scrapie). In addition, transmission experiments to primates suggest that some TSE agents other than the Classical BSE agent in cattle (namely L-type Atypical BSE, Classical BSE in sheep, TME, CWD agents) might have zoonotic potential. In particular, primates are highly permissive to L-type Atypical BSE, even by the oral route.

The opinion emphasizes that laboratory transmission experiments indicate that the L-type Atypical BSE agent has a significant zoonotic potential, which appears similar or even higher than that of the Classical BSE agent. While transmission data for evaluating the zoonotic potential of Classical scrapie in primates and human PrP transgenic mice are extremely limited or not yet available, a single study reported efficient transmission of a natural sheep Classical scrapie isolate to primates.

The opinion concludes that human PrP transgenic mice and primates are currently the most relevant models for investigating the human transmission barrier, but the extent to which such models are informative for measuring the zoonotic potential of an animal TSE under field exposure conditions is unknown. It is unpredictable whether a TSE agent will transmit to a new host, and if the transmission principally occurs, what the transmission rate will be.

Based on the results obtained with *in vitro* conversion assays, the opinion concludes that there is probably no absolute molecular barrier to transmission of TSE agents between mammalian species. Results also suggest that these assays may be developed as a tool for quantifying the transmission barriers between species for different TSE agent strains; however, there is no means at the moment to transpose *in vitro* results into the likelihood of *in vivo* interspecies transmission.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (TSEs) requires, since 2001, a comprehensive monitoring of TSEs in bovines, sheep and goats in the Member States. The testing results of ruminants for all TSEs (bovine spongiform encephalopathy, BSE, and scrapie) are collected in the database kept by the European Commission. In addition to the legal requirements, there has been a three-year EU survey related to Chronic Wasting Disease (CWD) in cervids, of which the data have also been collected in the Commission database.

The data concerning Creutzfeldt-Jakob Disease (CJD and vCJD, the new variant of CJD) cases in humans are collected in the national databases of the Member States. The European surveillance for CJD was established already in 1993 in the form of the European Collaborative Study Group of CJD (EUROCJD) in certain Member States (France, Germany, Italy, the Netherlands, Slovakia, Spain and the United Kingdom). Later, in 1997, Austria and some third countries (Australia, Canada and Switzerland) joined this group and the name was changed into the European and Allied Countries Collaborative Study Group of CJD. In this framework, the data from the national registries for CJD are compared between the countries with the objective to continue and further develop the surveillance of vCJD and to identify novel forms of CJD. In 1998, after the Council of the European Union recommended that epidemiological surveillance of CJD should be extended to all Member States (98/C 169/02), the NEUROCJD was established to cover more Member States (Belgium, Denmark, Finland, Greece, Ireland, Portugal and Sweden), as well as some more third countries (Iceland, Israel and Norway).

A great number of scientific studies have been carried out concerning the prion diseases, both in animals and humans. In addition there have been some epidemiological studies concerning the possible link between the animal and the human cases.

BSE is considered to be a zoonotic disease since vCJD has been proposed to be causally linked to BSE. For example in a review article “Variant CJD (vCJD) and Bovine Spongiform Encephalopathy (BSE): 10 and 20 years on: part 2” by J. G. Collee et al., published in *Folia Neuropathologica* (2006) 44/2, it is stated that biological and molecular strain typing studies have demonstrated clearly that the BSE agent and the vCJD agent are one and the same, or at least have a similar pathogenicity for man. Furthermore, the association between BSE and vCJD is supported by the epidemic curves, the presumed response to the various bans, the time-scales of the two diseases and their geographical occurrence. Already in an earlier study “Transmissions to mice indicate that “new variant” CJD is caused by the BSE agent” by M. E. Bruce et al, published in *Nature* (1997) 389 (6650), data provided strong evidence that the same agent is involved in both BSE and vCJD.

On the other hand, no direct scientific evidence of scrapie being a zoonotic disease has been presented. In this respect, reference is made to an article “Ten-year mortality from Creutzfeldt-Jakob disease in Cyprus” by S. Papacostas et al., published in *Eastern Mediterranean Health Journal* (2008) Vol 14, No. 3. The authors concluded that in Cyprus the CJD percentage was not higher than that expected according to global epidemiological surveillance, and no vCJD cases were found, despite the fact that scrapie has a very high incidence in the small ruminant population in Cyprus. A similar finding was made in an article “Scrapie of sheep and Creutzfeldt-Jakob disease in Iceland” by G. Georgsson et al., published in *Laeknabladid* (2008) 7-8, where the occurrence of CJD was studied over a period of 40 years (1960-2000). Although the Icelandic human population has been exposed to scrapie for 130 years, the article concluded that there is no indication that scrapie can be transmitted to humans and cause CJD. In an earlier article “Susceptibility of transgenic mice expressing chimeric sheep, bovine and human PrP genes to sheep scrapie” by A. Gombojav et al., published in *J. Vet. Med. Sci.* (2003) 65 (3), the results suggested that sheep strains of scrapie are not transmissible to humans.

The EFSA Scientific Panel on Biological Hazards adopted on 8 March 2007 a scientific opinion on certain aspects related to the risk of Transmissible Spongiform Encephalopathies (TSEs) in ovine and caprine animals (question EFSA-Q-2007-039). The opinion concluded that there is no evidence for an epidemiological or molecular link between classical and/or atypical scrapie and TSEs in humans, BSE being the only TSE agent identified as zoonotic. However, the transmissibility of other animal TSE agents to humans could not be excluded.

A comprehensive epidemiological study of TSEs in animals and humans at the level of the whole European Union has not been carried out so far.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

Based on the collected data on animal and human TSEs and the comprehensive review of the scientific publications or any other relevant data, the Commission requests the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) to provide a joint scientific opinion on any possible epidemiological or molecular association between the TSEs in animals (both classical and atypical strains) and humans.

ASSESSMENT

1. Introduction

1.1. Approach to the mandate

The zoonotic potential of animal Transmissible Spongiform Encephalopathies (TSEs) is an intriguing topic with considerable and far-reaching implications. Mankind has been in contact with TSEs in small ruminants for centuries if not millennia, but this issue has been seriously considered only since the emergence of Bovine Spongiform Encephalopathy (BSE) that has so far remained the only animal TSE with convincing scientific data on its transmission to humans in the form of variant Creutzfeldt-Jakob disease (vCJD). The history of emerging knowledge about the link between BSE and vCJD may serve as a lesson to learn how to properly identify a human risk from animal TSEs and to avoid mistakes in judgement. Thus one part of this document deals with the analysis of emerging scientific knowledge about the zoonotic character of BSE. However, as the prevalence of Classical BSE in the European Union (EU) is continuing to rapidly decline, it is now the emergence of atypical strains of BSE and scrapie, and possibly others in the future, that requires particular attention with regard to a potential public health risk. There is already a huge body of data of widely varying type available, in particular because of the rapid progress of molecular and bioassay studies, that need to be interpreted in an integrated way. Equally important, however, is recognition of the gaps in current knowledge. A balanced review may thus give a scientifically sound answer to the question on any possible link between animal and human TSEs.

Given the present wide mandate that encompasses consideration of *any* association between *any* TSE strain in animals and humans, the approach to this opinion by the EFSA Panel on Biological Hazards (BIOHAZ) and the European Centre for Disease Prevention and Control (ECDC) has to be equally comprehensive. It is considered essential to start with the definition of a zoonotic TSE, the means and ways how to identify it, what is relevant for a transmission barrier, which uncertainties are involved, and what is the role of TSE surveillance both in animals and humans. Subsequently, the approach of the opinion follows two lines. First, the available tools and methods need to be critically assessed, such as epidemiology, strain typing and risk modelling approaches using neuropathology, transmission experiments and *in vitro* laboratory assays, in particular their strengths and weaknesses with regard to relevance of their results for the mandate. Second, the considerable diversity of TSE agents must be individually reviewed with regard to the potential for transmission to humans. The review of available data for individual TSEs according to the methodological evidence, and of the uncertainties involved, provides the basis for scientifically based conclusions and recommendations.

1.2. Previous EFSA and ECDC assessments

Over the past years the European Food Safety Authority (EFSA) has been requested several times to provide scientific advice and in particular to perform risk assessments in the area of animal TSEs (i.e. BSE, Classical scrapie, Atypical scrapie and Chronic Wasting Disease (CWD) in ruminants), including specific questions on their zoonotic potential. In particular, EFSA's advice has been asked on some occasions by the European Commission in order to provide updated information on the zoonotic potential of TSEs other than BSE.

In 2007, EFSA, considering the available scientific information at that time, concluded that there was "*no evidence for an epidemiological or molecular link between Classical and/or Atypical scrapie and TSEs in humans*" and concluded that the BSE agent was "*the only TSE agent identified as zoonotic*" (EFSA, 2007a). However, in its opinion EFSA also highlighted a number of significant scientific uncertainties linked to this question. It indicated that the lack of association between TSEs in animals, in particular small ruminants, and humans may be biased by a number of factors, including the lack of

historical data on the prevalence of small ruminant TSEs, the lack of understanding of the true biodiversity of TSEs in small ruminants and in humans, as well as the predicted phenotype of disease that might arise should an animal derived TSE transmit to humans. That is why the EFSA opinion also concluded that in view of their diversity it was not possible to exclude transmissibility to humans of other animal TSE agents.

Following to a request for clarification on the above opinion by the European Commission, EFSA published, in 2008, an additional report on this question (EFSA, 2008b), also on the basis of further evidence that became available in the scientific literature. In its new document, EFSA clarified that, in ovine and caprine animals, no agents other than those causing BSE (only in caprine animals), Classical scrapie and Atypical scrapie had been so far identified. In addition, the report listed the evidence that did not allow excluding transmissibility to humans of TSEs other than BSE. This included some experiments leading to transmission of two Classical scrapie cases and one L-type Atypical BSE case to animal models of the human species (transgenic mouse and primate models). However, the same report also highlighted the existing limitations and uncertainties linked to the use of those models, in particular in relation to how well they represent the human species barrier and how well the experimental inoculation route employed represents exposure under natural conditions.

More recent EFSA opinions focused on the human exposure risk to TSEs through consumption of products deriving from small ruminants (ovine and caprine carcasses below six months, milk and milk products), but only focused on human exposure, without discussing the zoonotic potential of small ruminant TSEs (EFSA, 2008a, 2008c). A recent EFSA opinion (EFSA Panel on Biological Hazards (BIOHAZ), 2010a) provided updated data on the TSE infectivity distribution in small ruminant tissues. It also estimated the relative reduction of BSE infectivity load that can be achieved in the carcass of a small ruminant through the implementation of the current or alternative policies in terms of removal of Specified Risk Material (SRM). The zoonotic potential of TSE agents in small ruminants is, however, not discussed in the opinion.

A past EFSA opinion made an attempt to quantify the species barrier effect for BSE transmission from sheep to humans (EFSA, 2007b). This opinion considered that estimates for the barrier to transmission of BSE from cattle to humans, on the basis of epidemiological data, vary from 70:1 to more than 4,000:1. In the absence of experimental validation of these estimates, such figure should be considered with caution. In the case of BSE in sheep, a quantification was not possible according to the available epidemiological data, but lowering of the species barrier after passage through the bridging species (sheep) has been documented in experimental transmission studies in transgenic mice (Espinosa et al., 2007a; Espinosa et al., 2009; Plinston et al., 2010b).

In the past, EFSA and the former Scientific Steering Committee (SSC) of the European Commission were also asked to reflect on the zoonotic potential of CWD and on the possible role of cervids in the transmission of TSEs to humans. The SSC (2003) concluded that a theoretical risk for prion transmission to humans consuming products of CWD affected-cervids of all ages in countries where CWD exists cannot be excluded. The SSC also concluded that the early and widespread involvement of tissues in CWD infected animals did not allow defining a SRM list, neither to define any lower age cut off as has been defined for cattle in relation to BSE. Later on, an EFSA opinion (EFSA, 2004) concluded that even though human TSE-exposure risk through consumption of game from European cervids could be assumed to be minor, if at all existing, no final conclusion could be drawn due to the overall lack of scientific data. The opinion recognized a potential risk to consumers if a TSE would be present in European cervids and concluded that it might be prudent considering appropriate measures to reduce such a risk, e.g. excluding tissues such as central nervous system and lymphoid tissues from the human food chain, which would greatly reduce any potential risk for consumers. However, it also stressed that at that time no data regarding a risk of TSE infections from cervid products were available.

In 2009, several developments in the field of vCJD occurred:

- A case report described a 30 year old man who died in January 2009 with symptoms suggestive of vCJD. This individual had a genotype previously not associated with disease. This created concern that there may be a second wave of vCJD cases in humans with a different genetic background.
- The identification of a further possible vCJD infection in the spleen of a patient with Haemophilia A in the UK raised the possibility that vCJD infection can be transmitted from person to person through the use of plasma derived products.

Based on these developments, a number of questions were raised by the European Commission: should current assumptions on the number of people that may develop vCJD in the future be reviewed?; how does this impact on the current assumptions regarding transmissibility through blood transfusion and tissue/cells transplantation?; does this change the number of individuals at risk of developing vCJD following a transfusion/transplantation?; and are there measures to reduce any possible increased risk?

A rapid limited literature review was conducted and ECDC together with external experts prepared a rapid Risk Assessment (ECDC, In press). The heterozygote case described in December 2009 was formally classified as “possible” vCJD and there was some uncertainty this case indicated the start of a second wave of cases. Mathematical analysis of likely future case numbers in the UK and France, taking account of the potential wider genetic susceptibility, had already been carried out and was in the process of being published. The identification of potential transmission of vCJD through plasma derived products was a new finding calling for a review of assumptions on the number of people that may develop vCJD in the future. Although no clinical cases of vCJD had been connected to plasma derived products, there was a remaining theoretical risk that such products were able to transmit infection. However, the evidence for such transmission was limited. The transmission of vCJD by plasma derivatives had been predicted previously and preventive measures had been taken in regions with relatively high incidence of vCJD. The developments in 2009 added evidence that this prediction was true, but did not justify major changes in intervention strategies.

2. Definition and scientific basis

2.1. Zoonoses and TSEs

2.1.1. Definition of zoonosis

Several definitions of a zoonosis are available, some of which are reported here below:

- “Any disease and/or infection which is naturally transmissible directly or indirectly between animals and humans” (source: Directive 2003/99/EC⁶).
- “A disease or infection which is naturally transmissible from animals to humans” (OIE, 2010).
- “Any disease or infection that is naturally transmissible from vertebrate animals to humans and viceversa”⁷

Whatever definition is considered, it is clear that animals play an essential role in maintaining zoonotic infections in nature. Zoonoses may be bacterial, viral, or parasitic, or may involve prions (WHO/FAO/OIE, 2004). As well as being a public health problem, many of the major zoonotic diseases prevent the efficient production of food of animal origin and create obstacles to international trade in animal products.

Like many other infectious diseases, BSE clearly meets the definition of zoonotic disease. However, beyond this qualitative definition, a prioritisation in terms of the actual importance of animal TSEs for humans is helpful in order to produce interventions to TSE-associated threats and minimize public health risks. Several prioritisation approaches of infectious diseases in public health have been proposed which take several factors into account (Krause et al., 2008):

- burden of disease: incidence, severity, mortality;
- epidemiological dynamic: outbreak potential, epidemiological trend, emerging potential;
- information need: evidence for risk factors/groups, validity of epidemiological information, unknown pathogenesis;
- health-gain opportunities: preventability, treatability;
- disease/risk perception: political agendas and public awareness.

Priority rankings are not absolute but rather vary for different countries and parts of the world depending on the existent general health and hygienic standards. Priorities in South-East Asia, Africa or the Americas may differ from those in Europe.

In the EU the most important zoonotic agents are *Salmonella* spp., *Campylobacter* spp., verotoxigenic *E. coli* spp. and *Listeria monocytogenes*. Infections from those agents are non-lethal under most circumstances, albeit fatalities can occur. Other less common zoonoses include tuberculosis (due to *M. bovis*), Q-fever, borreliosis, brucellosis and infections with *Yersinia* spp., *Trichinella spiralis*,

⁶ Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC, as last amended. OJ L 325, 12.12.2003, pp.31-40.

⁷ Source: WHO website, available at www.who.int/zoonoses/en

Echinococcus spp., *Toxoplasma gondii*, *Cysticerci*, *Francisella tulariensis* and *Leptospira* spp. (EFSA/ECDC, 2010).

In contrast to the many bacteria associated zoonoses, indigenous viral infections play a minor role in Europe. Rabies is considered to be close to extinction and only Hantavirus, Tick-borne encephalitis and to a much lesser extent cowpox virus infections are receiving limited attention. Influenza and SARS may be considered as the only exceptions since the outcome of these pandemics/outbreaks were unpredictable at the beginning. It is a phenomenon that novel and/or emerging infectious agents alert the general public and receive scientific attention much more than already known “common” infectious agents. The first discovery of vCJD and the realization of the significant BSE exposure of the European consumers, in particular in the UK, ignited a political (and public) crisis in the EU. New threatening emerging viral zoonoses in the EU which have already entered the European continent are West Nile Virus (most mediterranean countries, Romania, Hungary, Austria) and Crimean Congo Hemorrhagic Fever infections (CCHF) (Balkan). Numbers of human fatalities are comparable to vCJD numbers (i.e. approx. 200 CCHF deaths in Europe in the last decade). Others like Rift Valley Fever, Henipa and Ebola viruses infections can be introduced into the EU.

A discussion on the details of the BSE epidemic comes later in the document. Measures that were introduced in the EU over the past 20 years have been aimed at minimising human exposure to BSE. However there had been significant human exposure to BSE infectivity in the food chain, particularly in the UK. The scale of the associated vCJD epidemic was unpredictable at the beginning, but much lower case numbers of vCJD have been found than originally feared, indicating that infection protection measures for humans and animals have been effective. However, due to the protracted incubation periods in combination with the absence of preclinical tests for BSE infection in live humans or animals, considerable uncertainty remains.

2.1.2. Principles for the identification of zoonotic diseases

A zoonotic potential may be indicated by epidemiological studies, and evidence supporting an association may be derived from laboratory studies. The following general statements regarding zoonoses are extracted from the report of a WHO/FAO/OIE Joint Consultation on Emerging Zoonotic Diseases (WHO/FAO/OIE, 2004).

“There are a number of general guiding principles for studying zoonotic diseases:

- *identify the source of infection, to determine whether it is from wildlife, domestic or peridomestic animals, or from multiple sources;*
- *establish the mode of transmission, to determine whether it is by direct contact, vector-borne, environmental contamination, or a combination of modes;*
- *identify potential host species and the natural reservoirs of the zoonotic pathogen. Current molecular and epidemiological knowledge can be used to identify target species for surveys;*
- *conduct preliminary surveys of target species and follow-up, when indicated, with long-term ecological and epidemiological studies of identified reservoir species in the wild and/or in an experimental setting where appropriate.*

[...] The following elements are considered important for surveillance or early warning and alert systems for emerging zoonotic diseases.

Syndromic surveillance of humans and animals

Such surveillance should:

- *be representative of the population under surveillance;*
- *detect unusual clusters of morbidity and mortality in space and time;*
- *be species, region and disease dependent;*
- *be based on sentinel surveillance;*

[...]

- *use both passive and active surveillance as appropriate.*

Syndromic surveillance of animals

In addition to the above, syndromic surveillance of animals should also include:

- *decreases in production (e.g. milk or egg);*
- *both domesticated and wild animals.*

[...] *No emerging animal disease should be neglected and such diseases should always be monitored and assessed for their public health impact. [...] Data on zoonoses and zoonotic agents in animals, food, feed and humans listed in the EU's Zoonoses Directive (2003/99/EC) need to be collected at the national level and communicated to the ECDC and EFSA".*

One means of assessing the potential causal links between animal and human TSEs is to apply the Bradford Hill guidelines (Bradford Hill, 1965), which continue to be used in public health to assess the strength of causal relationships in epidemiology. The guidelines include consideration of the following criteria: strength, consistency, specificity, temporality, biological gradient, biological plausibility, coherence, experiment and analogy. Not all criteria in the guidelines need to be fulfilled to suggest a causal link and some of the criteria are now judged to be less important than others, notably analogy and consistency. For disease with a long latency, temporality is of significant importance.

2.1.3. The challenges in identifying a TSE as a zoonosis

The conventional approach to identify a human TSE as zoonotic has been to search for atypical cases of human TSEs in populations and this remains an important goal with the advent of atypical animal TSEs. However, the issue of the aetiology of already existing human TSEs must also be kept under review. Recently there have been publications suggesting a possible link between animal and human TSEs based on laboratory studies, including transmission studies and/or analysis of the characteristics of brain prion proteins, rather than starting from the clinical/epidemiological evidence (Beringue et al., 2008a; Comoy et al., 2008b; Zanusso, 2010). An important challenge is to interpret this type of evidence if this occurs in isolation from epidemiological evidence.

In TSE diseases, the lack of specific nucleic acids associated to the infectious agent makes the traditional phylogenic approaches usually applied for identification of infectious agents inadequate.

The combined analysis of human and animal epidemiology is an essential approach for assessing a possible relationship between human and animal disease. However the reliability of such approach for identifying a "zoonotic link" is strongly dependent on the nature and the quality of the available data.

In that respect, TSE diseases in both animals and humans have specific characteristics which must be considered. In particular:

- the low incidence of identified CJD cases (1-1.5 case per million of the general population and per year) which impacts on the power of the analysis;
- the extended incubation period in acquired forms of human TSEs (which can reach several decades), that impairs the reliability of retrospective investigation on exposure of patients to potential risk factors;
- the limitations of the epidemiology-surveillance (passive or active) of animal TSEs (as illustrated by the recent discovery of Atypical scrapie in small ruminants, and L-type and H-type Atypical BSE in cattle);
- the global trading system and travel, which complicate the analysis of exposure risk;
- if only one of the many non-BSE TSE strains in ruminants has zoonotic potential, this may be difficult to identify.

The key event in TSE is the conversion of a normal cellular protein (PrP^C) into an abnormal isoform which polymerises/self-aggregates and accumulates in tissues in infected individuals (McKinley et al., 1983). According to the prion concept, abnormal PrP is the causative agent of TSEs (Prusiner, 1982).

Historically, abnormal PrP was operationally defined as insoluble and resistant to protease digestion (Bolton et al., 1982) and was subsequently termed PrP^{res} (or PrP^{Sc}). While normal PrP protein is soluble in detergents and sensitive to proteinase K (PK) digestion, part of PrP in infected tissues is PK-resistant and is recovered in the pellets of centrifuged samples.

While PrP^{res} aggregates/polymers have been shown to be infectious (Prusiner, 1992), it is unclear if the totality of the infectious particles is entirely congruent/associated with these abnormal forms/species (Aguzzi et al., 2008; Soto and Castilla, 2004; Tixador et al., 2010). Indeed PrP^{res} aggregates are not the only abnormal forms detected in diseased tissues, as exemplified by the detection of a substantial fraction of disease-associated, PK-sensitive forms of PrP (Caughey et al., 2009; Pastrana et al., 2006; Safar et al., 1998; Safar et al., 2005; Thackray et al., 2007; Tzaban et al., 2002), the infectivity of which remains to be determined. Moreover an efficient transmission in the absence of detectable PrP^{res} has been observed in different experimental models of TSE diseases, in particular following cross-species transmission events (Barron et al., 2007; Lasmezas et al., 1997; Zou et al., 2010). Currently, field monitoring of TSEs relies on the application of tests that detect PK-digested and/or large/sedimentable PrP^{Sc} forms. Beyond the question of the analytical sensitivity of these tests (the capacity to detect minute amounts of PrP^{res}), the possibility that infectivity might dissociate from PrP^{res} in some situations raises some conceptual concerns on the efficiency of the detection of TSE infected individuals or animals.

The characterization of TSEs ultimately relies on the detailed phenotype of the disease after its propagation in animals, humans and model systems (bioassay). An indication that the same agent may be the cause of a TSE in two different hosts is given by the comparison of laboratory phenotypes. Apart from the material constraints (time, cost etc.), this approach can also suffer from several intrinsic drawbacks, and despite forty years of continuous work and improvements (animal models-phenotyping approach) our comprehension of the true diversity of the TSE agents and our capacity to describe this remains incomplete.

2.1.4. The BSE-vCJD example

The BSE agent has been recognised as a zoonotic agent, causing vCJD in humans. The means by which BSE and vCJD were linked may have lessons for the identification of other zoonotic TSE diseases.

Following the emergence of BSE in the UK in the 1980s epidemiological surveillance of CJD was initiated to determine whether there was any change in the characteristics of CJD that might indicate a link with BSE. The phenotype of any BSE related human disease could not be predicted and a surveillance system was established which specifically sought to identify all cases of human TSE disease, including those with atypical characteristics. Potential indicators that might indicate that a novel TSE disease had developed were considered to include:

- i) A change in the phenotype of cases: e.g. clinical characteristics, results of specialist investigations, neuropathology. This presumed that infection with an animal TSE, such as BSE, would result in a change in the clinico-pathological phenotype that could be distinguished from already existing human TSEs. It was recognised that the identification of a human TSE as a zoonotic disease would be difficult in the absence of a change in phenotype.
- ii) A change in demographic characteristics: e.g. age, sex incidence. This also presumes that infection with an animal TSE might result in a change in either of these parameters. The effect of a zoonotic TSE on these parameters cannot be predicted.
- iii) A change in epidemiological features: e.g. incidence and mortality rates, geographical distribution. Because of the known variability in incidence and mortality rates in human TSEs, only a major outbreak of a zoonotic TSE could be identified by assessing a change in these parameters. The known variation in the geographical distribution of BSE and therefore the risk to human populations indicated that a change in the characteristics of human TSEs in the country at greatest risk of human exposure, the UK, in comparison to other countries might indicate the development of a novel zoonotic disease in humans. In animal TSEs with a relatively homogenous geographic distribution or in which the true distribution had not been established, this strategy would be ineffective.
- iv) Identification of novel risk factors for disease: e.g. occupation, diet, medical exposures. There were a range of potential mechanisms of transmission of BSE to the human population and these were assessed by obtaining detailed information on a range of parameters in each incident case of human TSE. This information was obtained prospectively in all cases because of the possibility that a zoonosis might only be identified through a change in these parameters.
- v) Laboratory confirmation of a common infectious agent: e.g. biochemical analyses of PrP, laboratory transmission studies.

The identification of a change in any of these parameters assumed systematic ascertainment of cases of CJD, accrual of detailed information on cases and the availability of baseline data to identify a change from that expected. The use of historical data on CJD in the UK as a baseline was potentially compromised by an improvement in case ascertainment in the first years of the UK CJD surveillance programme and a collaborative study on the epidemiology of CJD in a number of EU countries was initiated in 1993 and proved to be critical to the identification of a link between BSE and vCJD in the UK.

A new type of CJD (vCJD), potentially causally linked to BSE, was identified in the UK in 1996. A detailed description of the process of the recognition of vCJD was provided to the BSE Inquiry⁸. In brief the following factors were critical to the appropriate interpretation of the significance of a cluster of atypical cases of CJD identified in the UK in 1995-1996:

⁸ The BSE Inquiry, available at <http://collections.europarchive.org/tna/20090505194948/http://bseinquiry.gov.uk/report/index.htm>

i) To what extent are these cases different and new?

a. Is there a novel clinical phenotype?

vCJD had a distinct mode of onset with prominent psychiatric symptoms, often associated with persistent painful sensory symptoms, and a duration of illness more protracted than in sporadic CJD (sCJD) (Spencer et al., 2002). The age of patients was significantly younger than in sCJD and the EEG did not show the characteristic periodic complexes seen in sCJD (Zeidler et al., 1997).

b. Is there a novel neuropathological phenotype?

The neuropathological changes in vCJD were novel with extensive deposition in the brain of florid plaques in addition to the expected spongiform change, neuronal loss and astrocytic gliosis. The novelty of the neuropathological changes was confirmed by subsequent investigation (Ironsides et al., 2000).

c. Are these cases linked to *PRNP* mutations?

Sequencing of the prion protein gene (*PRNP*) in all cases excluded mutations of *PRNP*.

d. Is there ascertainment bias or increased efficiency of surveillance?

The possibility that these new cases reflected improved ascertainment rather than a new disease was of concern because of the evidence of an increase in the mortality rates for sCJD in the UK. Comparative data on CJD from countries with a similar potential bias was essential to assess whether this new disease was occurring only in the UK, and at the time vCJD was identified, no cases with a similar phenotype had been identified in other countries.

ii) To what extent might these cases be linked to BSE?

a. Are these cases in the UK distinct from previous experience?

The availability of systematic data on CJD in the UK from 1970 onwards allowed a review of the demographic, clinical and neuropathological data, which indicated that cases with this phenotype had not been identified prior to 1995 and that a novel risk factor for human TSE disease might be present in the population.

b. Are these cases only occurring in the UK?

The hypothesis of a link with BSE depended in part on demonstrating that this new form of CJD was occurring in the country with the greatest risk of human exposure to the BSE agent, the UK, and not with the same frequency in countries at significantly lower risk. In 1996 data on cases of sCJD in younger age groups (aged less than 50 years) was provided by a number of continental EU countries and this indicated that cases with a similar novel phenotype had not been, at that time, identified in the other countries, consistent with the hypothesis of BSE as the novel risk factor for human disease.

Evidence from laboratory studies was essential to provide support to the hypothesis of a causal link between BSE and vCJD, which had been raised by the epidemiological and clinico-pathological findings. The biochemical form of prion protein deposited in the brain in vCJD patients was found to be indistinguishable from that in BSE, the neuropathological changes in macaques inoculated with BSE was similar to that of vCJD and transmission studies in laboratory rodents showed that the characteristics of the infectious agent in BSE and vCJD were remarkably similar (Bruce et al., 1997; Scott et al., 1999). All together, this was consistent with the hypothesis that the two diseases were

caused by the same TSE agent. Subsequent epidemiological and laboratory evidence has confirmed that BSE is a zoonotic disease causing vCJD in humans.

In order to further support the causal relationship between vCJD and BSE, it may be tested within a causal framework that makes the judgement explicit. As mentioned above, a commonly used set of guidelines was proposed by Bradford Hill (1965). Although the limitations of the mechanical application of such an approach or the weakness of some of the listed criteria of the guidelines have been underlined (Bhopal, 2008; Rothman et al., 2008; Szklo and Nieto, 2007), it may be helpful to follow this approach in explaining how the association observed between the two diseases may be considered of causal nature.

In relation to the Bradford Hill guidelines the causal association between BSE and vCJD can be rated as follows (taking in mind that Bradford Hill (1965) stated: “None of my nine viewpoints can bring indisputable evidence for or against the cause-and-effect hypothesis and no one can be required as a *sine qua non*”):

1. Strength (does exposure to the supposed cause raise the incidence of disease?). The incidence of vCJD is highest in the country (UK) with the greatest potential exposure to BSE. Moreover: (i) in a cases-control study (Ward et al., 2006) a frequent consumption of beef and beef products thought likely to contain mechanically recovered or head meat, or both, including burgers and meat pies, was associated with increased risk for vCJD; (ii) in an ecological study, the occurrence of vCJD outside the UK has been correlated with the level of live bovines or carcass meat imported from the UK (Sanchez-Juan et al., 2007).
2. Consistency (is the association between supposed cause and outcome consistent across different studies and between subgroups?). The occurrence of vCJD in countries in which the human population were also exposed to BSE is consistent with the hypothesis of a causal link between BSE and vCJD.
3. Specificity (is the association within the supposed causes specific to relevant diseases and are diseases associated with a limited number of supposed causes?). The clinical and pathological phenotype was novel and unique; the geographic distribution of cases of vCJD is consistent with a link with BSE whereas no correlation has been shown with other animal TSEs such as scrapie or CWD.
4. Temporality (does the supposed cause precede the disease?). Bradford Hill (1965) indicated that “this is a question which might be particularly relevant with diseases of slow development”. The occurrence of vCJD ten years after the identification of BSE is consistent with known incubation periods in acquired human TSEs.
5. Biological gradient (i.e. dose response: does varying exposure to the supposed cause lead to varying amounts of disease?). There is evidence from the quoted case-control study of increased risk of vCJD in relation to increased dietary consumption of bovine products containing high titres of infectivity.
6. Biological plausibility (is there a biological mechanism by which the supposed cause can induce the effect?). The possibility that BSE could result in a human disease resulted in extensive measures to minimise human exposure to the BSE agent in the UK prior to the identification of vCJD. Moreover the efficacy of the exposure via the oral route and the possibility for TSE agents to cross species barriers have been shown in a number of experimental transmission studies.
7. Coherence (is there any conflict between the causal interpretation and the known natural history and biology of disease?). The neuropathological changes in vCJD are novel and consistent between cases.

8. Experiment (any related research which makes a causal inference more plausible). The infectious agent in vCJD is indistinguishable from the agent of BSE in experimental studies, including molecular strain-typing (Hill et al., 1997) and experimental transmission to mice (Bruce et al., 1997; Scott et al., 1999) and primates (Lasmezaz et al., 1996).
9. Analogy (an accepted phenomenon in one area that can be applied to another area). Kuru, scrapie and CWD are analogous as these TSE diseases are sustained by oral exposure to similar infectious agents. Moreover transmissible mink encephalopathy (TME) developed in United States ranches as the result of the consumption by the animals of ruminant carcasses (Marsh and Hadlow, 1992). However, as suggested by Rothman et al. (2008), analogy is a very weak criterion.

There is no evidence that the BSE-vCJD association is due to the effect of confounding, bias or chance. Bradford Hill (1965) comments on tests of significance that “*no formal tests of significance can answer these questions [...] Such tests can, and should, remind us of the effects that the play of chance can create, and they will instruct us in the likely magnitude of those effects. Beyond that they contribute nothing to the ‘proof’ of our hypothesis*”.

It is important to stress that there was an evolution of knowledge in relation to the causal link between BSE and vCJD, and Table 1 below summarises an assessment of the criteria in the Bradford Hill guidelines at various time points in relation to this hypothesis. It is important to stress that in relation to these timings BSE itself was first identified in 1986 and vCJD in 1996.

Table 1: Criteria of the Bradford Hill’s guidelines and assessment of the BSE/vCJD link with time.

	Jan 1996	Mar 1996	1997	2000	2006
1. Strength		+	+	+	+
2. Consistency				+	+
3. Specificity		+	+	+	+
4. Temporality	+	+	+	+	+
5. Biological gradient					+
6. Plausibility	+	+	+	+	+
7. Coherence		+	+	+	+
8. Experiment			+	+	+
9. Analogy	+	+	+	+	+

Conclusions:

- The process by which BSE was identified as a zoonosis indicates that there are a number of essential components when identifying an animal TSE disease as causing disease in the human population:
 - The facility to diagnose human TSE disease in populations, in particular to identify new forms. This requires systematic ascertainment of all suspect cases of human TSEs through epidemiological surveillance, accrual of details of clinical and demographic characteristics, access to expert neuropathology with preferably a high *post-mortem* rate, and access to specialist investigations, including prion protein gene sequencing.

- Background information on the characteristics of known forms of human TSE disease in populations, including mortality rates and phenotypic characteristics to allow the recognition of potentially novel findings.
- Detailed information on the epidemiology of animal TSE diseases to allow assessment of the potential implications of novel findings in human disease and to guide the focus of human epidemiological enquiry.
- Access to laboratory studies, including transmission experiments, to assess the agent strain in specific unusual cases of human TSE disease which are identified through epidemiological surveillance systems.

2.2. Diversity of TSE agents, strain definition and transmission barrier

2.2.1. Defining a TSE strain

Definitions of isolate, strain and cloned strain are provided here below:

- An “isolate” is a primary source of TSE from the natural disease. It may be passaged in the natural host or in another species. It may contain one or more “strain”.
- A “strain” refers to a source which has been characterised phenotypically in a host and which behaves as a single entity within that host, as far as can be demonstrated experimentally. Several serial passages in the same host species and PrP genotype are usually required to establish the phenotypic properties of strains.
- A “cloned strain” refers to a strain which has been subjected to serial passage at limiting dilution, with the aim of diluting out any minor, slower replicating strains in the recipient.

While some aspects of the characterisation of TSE agents are conceptually similar to approaches used for other micro-organisms in that differences in a range of phenotypic properties are measured, the lack of specific nucleic acids associated to the infectious agent is a significant challenge and makes the traditional phylogenetic approaches usually applied for identification of infectious agents inadequate.

Historically, strain typing was carried out using laboratory rodent models, predominantly wild type mice, in which a range of characteristics were assessed to define strains. Most commonly these can include relative incubation periods, clinical signs, the amount and distribution of vacuolar pathology and in some cases other pathological features (lesion profiling), and, more recently, differences in the biochemical properties of PrP^{Sc} (molecular profiling) and PrP^{Sc} distribution in brain and peripheral tissues. Strain discrimination is dependent on the number and discriminatory power of these measurements.

A substantial proportion of human and animal TSE isolates cannot be propagated into conventional mice models, which limits the usefulness of this system to characterize and compare TSE agents circulating in the field. Moreover the propagation of natural TSE isolates into inbred mice lines implies the passage across a species barrier (See below), which may impact on the properties of TSE agents. Crossing a species barrier may select for different strains that can replicate in the recipient species. Such strains may not represent the dominant populations in the donor.

Strain typing is feasible in other species but is limited by the lack of inbred animals differing in their PrP genotype. Both the hamster and bank voles have proved useful species for differentiating between a number of TSE strains.

More recently transgenic mice with PrP derived from different species have been increasingly used in typing TSE agents which do not readily transmit to wild type mice (Groschup and Buschmann, 2008). Advantages and disadvantages of various transgenic mice models are discussed later in the document.

Conclusions:

- Currently TSE agent characterisation mainly relies on experimental transmission in animal models.
- Strain typing, by measuring a range of properties of TSE agents, has succeeded in characterising a variety of distinct agents including the BSE and vCJD agents.
- Multiple tests in different animal models are recommended for any strain typing.

2.2.2. Diversity of natural TSE strains

Multiple strains of TSEs may exist in a single host. The genetic background of the host can influence the strain characteristics, and moreover when a strain moves between species, strain characteristics can alter in unpredictable ways (Bartz et al., 1998). We are very dependent on the limited experimental tools available for strain characterisation and the limits imposed by these tools in defining strains and the relationship between these strains.

2.2.2.1. Sheep strain diversity

Scrapie was discovered more than 200 years ago in the UK. However the precise number of strains behind this generic term used to designate sheep and goat TSE has remained so far elusive. An “atypical” form of scrapie has been newly described in European flocks through active surveillance programs based on the rapid detection of PrP^{res} in animal brain tissues at the abattoir/slaughterhouse.

The existence of 26 major polymorphisms in the sheep *PRNP* is a factor that is likely to impact the description of sheep scrapie strain diversity. At least three distinct types of scrapie agents have been identified/isolated in the natural host: SSBP/1 (Dickinson et al., 1968b), CH1641 (Foster and Dickinson, 1988) which both have very similar characteristics to natural scrapie isolates, and Atypical scrapie agent (Benestad et al., 2003; Simmons et al., 2007). Many differences in pathological outcome (Jeffrey and Gonzalez, 2007) suggests further strains may exist within natural scrapie. Moreover, multiple strains may exist in a single host. If there is any relationship between sheep scrapie strains it has yet to be defined. To date, natural BSE has not been reported in sheep, although experimentally sheep are highly susceptible to the BSE agent.

Historically, appreciation of potential scrapie diversity has relied on serial passaging of natural isolates to a panel of inbred mouse lines, where strains that preferentially replicate in the mouse are isolated and comparison of the biological phenotype obtained (Bruce, 2003). The considerable strain variations initially described (up to 20 strains, (Bruce and Dickinson, 1987; Dickinson, 1976)) might finally be restricted to a varying combination of 3 distinct strains, as far as UK field scrapie cases are concerned (only 20 isolates studied): ME7 and 87A in *Prn-a* mice, and 87V in *Prn-b* mice (Bruce, 1993, 2003; Bruce et al., 2002). The more recent transmission of 10 UK scrapie cases identified again ME7 and possibly a new strain, termed 222C (Bruce et al., 2002). The relationship between these mouse-adapted TSE agents and those naturally present in the TSE isolates transmitted is not clear, as new strains may have emerged after crossing the transmission barrier. Another shortcoming of this methodology is that wild-type mice are refractory to TSE from many but not all sheep homozygous for the ARQ allele (Bruce, 2003; Bruce et al., 2002; Foster and Dickinson, 1988; Horiuchi et al., 2002) and from atypical cases (Bruce et al., 2007; Griffiths et al., 2010; Le Dur et al., 2005). An additional shortcoming is the lack of standardisation of strain definition in other models.

The use of a particularly susceptible host, such as bank voles, or the combination of transgenic mouse lines expressing ovine (or caprine) *PRNP* genes may permit a more comprehensive view of strain diversity in small ruminants in the near future because of a lower transmission barrier. As a striking example, Atypical scrapie cases can propagate without apparent species barrier in “ovinizated” transgenic mice overexpressing sheep VRQ allele (Le Dur et al., 2005). The diversity of Classical scrapie agents is being explored in this model. Serial transmission of about 80 isolates from Europe has so far permitted to categorise these isolates in four phenotypically distinct classes (Beringue et al., 2007; Beringue et al., 2006; Le Dur et al., 2005; Thackray et al., 2008; Tixador et al., 2010; Vilotte et al., 2001). The molecular behaviour of sheep scrapie isolates in ovine transgenic mice expressing another PrP allele (ARQ mice, (Crozet et al., 2001)) was also variable, suggesting the existence of distinct strains. Some exhibited features similar to CH-1641 (~19 KDa PrP^{res} signature). In other, ~21 KDa forms predominated and there was a mixture of strains (Baron and Biacabe, 2007; Baron et al., 2004).

Conclusions:

- There are multiple strains of sheep scrapie, including Atypical scrapie, that can be identified by passage through sheep, wild type and transgenic mice.
- The diversity of TSE agents occurring in ovine animals cannot be considered to be fully characterised.
- Multiple strains may exist in a single host.
- To date, natural BSE has not been identified in sheep.

2.2.2.2. Goat strain diversity

From the clinical and pathological observations of natural goat scrapie, it has to be assumed that there is more than one strain. Historical evidence for strains is found in an observation from the passage of sheep brain pool SSBP/1 into goats. Two phenotypes were seen, “drowsy” which most likely was derived from a natural infection, and “scratching” which came from the challenge. Subpassage into mice of isolates from these two goat phenotypes resulted in different strains (Dickinson, 1976). Strain typing of current goat scrapie cases has only been initiated recently and bioassay data to estimate the number and characteristics of strains are not yet available. To reflect the European biodiversity of scrapie strains in goats a number of natural isolates from different countries has been selected for testing in transgenic mice with high susceptibility for ruminant scrapie. These isolates were pre-screened for the biochemical characteristics of abnormal PrP and selected so that they represented the maximum number of PrP types (Bossers et al., 2006). It is to note that goats with Atypical scrapie (Le Dur et al., 2005) and BSE (Eloit et al., 2005; Jeffrey et al., 2006) have been identified in Europe through active surveillance programs.

Conclusions:

- There are potentially multiple strains of goat scrapie.
- The diversity of TSE agents occurring in caprine animals cannot be considered to be fully characterised.
- Atypical scrapie has been identified in goats.
- BSE has been identified in goats.

2.2.2.3. Cattle strain diversity

There is good evidence to indicate that a single agent has been responsible for the BSE epidemic, although after laboratory passage at least two strains have been isolated (Bruce et al., 1997).

The large-scale testing of livestock nervous tissues for the presence of PrP^{Sc} has led to the recognition, in Europe, Japan and the USA, of two molecular signatures distinct from BSE in rare cases of aged cattle, consistent with the possibility of sporadic forms of TSE in cattle (Biacabe et al., 2008). These Atypical BSE agents were termed H-type Atypical BSE (H-BSE) and L-type Atypical BSE (L-BSE or BASE), respectively. Their PrP^{Sc} molecular signature differed from Classical BSE in term of protease-resistant fragments size and glycopattern (Biacabe et al., 2004; Buschmann et al., 2006; Casalone et al., 2004). In addition, L-BSE has a tendency to form amyloid plaques in cattle brain and has a distribution of brain pathology distinct from Classical BSE (Casalone et al., 2004).

The experimental transmission of H-BSE cases to different lines of bovine PrP transgenic mice unambiguously demonstrated their infectious nature. The initial transmissions of H-BSE produced a distinctive strain phenotype as compared to Classical BSE (Beringue et al., 2007; Beringue et al., 2006; Buschmann et al., 2006; Capobianco et al., 2007).

L-BSE also retained unique and distinct phenotypic features, compared to Classical BSE, upon transmission to bovine PrP transgenic mice (Beringue et al., 2007; Beringue et al., 2006; Buschmann et al., 2006; Capobianco et al., 2007).

Conclusions:

- A single agent has been responsible for the Classical BSE epidemic.
- At least three TSE agents have been identified in cattle.
- The diversity of TSE agents that might occur in bovine animals cannot be considered to be fully characterised.

2.2.2.4. Cervid strain diversity

Chronic wasting disease (CWD) is a TSE affecting free-ranging and captive mule deer (*Odocoileus hemionus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*, referred to as elk in the text below) and Shira's moose (*Alces alces shirasi*) (Sigurdson, 2008). Documenting strain variation of CWD agents is complicated by the multiplicity of affected species. First discovered in northern Colorado in 1967, the disease has expanded, and currently has been recognized in 15 US states and 3 Canadian provinces. While its origin remains enigmatic, several aspects of its physiopathology are reminiscent of sheep scrapie. CWD has not been reported outside North America, except from exported disease into South Korea. BSE has not been reported in cervids.

CWD does not readily transmit to wild type mice (Browning et al., 2004). The last few years have witnessed the development of several transgenic mouse lines expressing either elk or deer PrP (Browning et al., 2004; Kong et al., 2005; LaFauci et al., 2006; Tamguney et al., 2006), including mice expressing polymorphisms at codon 132 or 96 (Green et al., 2008b; Meade-White et al., 2007). Although some studies raised the possibility of CWD strain variation (Browning et al., 2004; LaFauci et al., 2006), the limited number of isolates and the lack of detailed strain analyses meant that this hypothesis remained speculative. Subsequent studies supported the feasibility of using transgenic mice for characterizing naturally occurring CWD strains, CWD prions generated by protein misfolding cyclic amplification (PMCA), and novel cervid TSE agents (Green et al., 2008c). Comparative studies of CWD in transgenic mice expressing deer and elk PrP (Angers et al., 2009) also identified residue 226, the sole primary structural difference between deer and elk PrP, as a major

determinant of CWD pathogenesis, and supported the different clinical and pathological properties of CWD in these species. Recently, the prevalence of CWD strains in a large collection of captive and wild cervids from different species and geographic locations was assessed by bioassay in transgenic mice (Angers et al., 2010). The findings provided substantial evidence for two prevalent CWD prion strains, referred to as CWD1 and CWD2, with different clinical and neuropathological properties. Transmissions from elk favored either CWD1 or CWD2 profiles, while transmission of deer inocula favored the production of mixed intra-study incubation times and CWD1 and CWD2 neuropathologies. These findings indicate that elk may be infected with either CWD1 or CWD2, while deer brains tend to harbor CWD1/CWD2 strain mixtures.

Additional previous studies also support the existence of multiple CWD strains. CWD has also been transmitted, albeit with varying efficiency, to transgenic mice expressing mouse PrP (Sigurdson et al., 2006; Tamguney et al., 2006). In the former study, a single mule deer isolate produced disease in all inoculated Tga20 mice. On successive passages, incubation times dropped to ~ 160 d. In the second study, 1 elk isolate from a total of 8 deer and elk CWD isolates induced disease in 75 % of inoculated Tg4053 mice. It is worth noting that the distribution of lesions in both studies appeared to resemble the CWD1 pattern. Low efficiency CWD prion transmission was also recorded in hamsters and transgenic mice expressing Syrian hamster PrP (Raymond et al., 2007). In that study, during serial passage of mule deer CWD, fast and slow incubation time strains with different patterns of brain pathology and PrP^{Sc} deposition were also isolated.

Conclusions:

- There are at least two different strains causing CWD.
- The diversity of TSE agents occurring in cervids cannot be considered to be fully characterised.

2.2.2.5. Human strain diversity

The shared characteristics of BSE and vCJD isolates in most experimental animal models provide compelling evidence for a common causal agent. A number of forms of human TSE have been transmitted in laboratory studies, including both sporadic and genetic forms of disease, but the relative resistance of wild type mouse models has limited the ability to characterise specific agent strains. There is, however, evidence of strain variation in sCJD based on laboratory studies in bank voles (Nonno et al., 2006) and transgenic PrP mice (Bishop et al., 2010).

Conclusions:

- There are multiple strains of human TSEs.
- The shared characteristics of BSE and vCJD isolates in most experimental animal models provide compelling evidence for a common causal agent.

2.2.3. Capacity of agents to cross the transmission barrier

Intra-species transmission of a TSE agent was first demonstrated with sheep scrapie in the 1930s' (Cuillé, 1936). Cross-species transmission is generally less efficient as evidenced by extended incubation period and incomplete attack rate at primary passage. This so-called "species barrier" (Pattison and Millson, 1961b) is usually abrogated after a few subpassages, reflecting the adaptation of the agent to its new host. The species barrier may be overcome by changes in the properties of the infecting agent, by mutation, selection of faster replicating species, higher effective titres and routing effects. In seminal experiments, it was established that distinct strains could be raised and propagated in different lines of inbred mice upon serial adaptation of sheep or goat scrapie isolates (Dickinson, 1976; Dickinson et al., 1968a). However, the parameters controlling both intra- and inter-species

transmission of TSE agents are not well defined. Both the PrP gene sequence of the host and the TSE strain appear to be equally important in determining the issue of a cross-species transmission but an understanding of how these components contribute is not sufficient to allow predictions of cross-species transmission.

2.2.3.1. The PrP gene and protein

Both PrP sequence and tertiary PrP^C structure (Wüthrich and Riek, 2001) are highly conserved among mammalian species. The highly variable susceptibilities of sheep breed to experimental or natural scrapie indicate that minimal amino acid divergences in the PrP sequence may have a major impact on the transmission efficiency (Belt et al., 1995; Elsen et al., 1999). Polymorphism at residue 129 (M or V) and other polymorphisms of human *PRNP* similarly influences incubation times and/or susceptibility to sporadic, acquired and variant forms of CJD (Bishop et al., 2006; Collinge, 2001; Wadsworth and Collinge, 2007). Experimentally, polymorphisms and mutations in the murine *PRNP* gene have been shown to alter transmission barriers between species (Barron et al., 2001).

Early studies suggested that the cross-species barrier resides essentially in PrP primary structure differences between the host and donor species. In seminal transgenesis experiments, the recognized resistance of the mouse to hamster scrapie (Kimberlin and Walker, 1978) was abrogated by expressing hamster PrP^C in mice (Scott et al., 1989). This apparent lack of species barrier has led to the development of a long list of mouse lines expressing the mammalian PrP sequence of interest, either by additional transgenesis or by a gene replacement method (See Appendix A).

Conclusions:

- The parameters controlling both intra- and inter-species transmission of TSE agents are not well defined.
- PrP sequence is one important factor in determining cross-species transmissions. However it is not possible through simple PrP sequence analysis to predict cross-species transmissions.
- Transgenic expression of a foreign PrP gene in mice has been a powerful means to model naturally occurring TSE diseases by abrogating the species barrier.

2.2.3.2. Role of the TSE strain

The TSE strain plays a pivotal role in cross-species transmission events. This has been brought to light by the apparent capacity of the BSE agent to transmit to other species such as exotic ruminants, cats and humans (Bruce et al., 1997; Collinge et al., 1996; Scott et al., 1999). Similar observations have been obtained with other TSE agents. For example, sporadic and genetic CJD cases can be experimentally propagated in bank voles with a low transmission barrier despite the divergence between human and vole PrP sequences (Nonno et al., 2006). Strikingly the incubation time observed on primary inoculation to voles was similar to that in transgenic mice overexpressing human PrP (Asante et al., 2002; Beringue et al., 2008c). Parallel transmission of the same CJD isolates to inbred mice was inefficient (Nonno et al., 2006), consistent with earlier transmission data (Bruce et al., 1997; Gibbs and Gajdusek, 1973; Hill et al., 1997). Transmission to mice of vCJD isolates was efficient (Bruce et al., 1997), whereas transmission to bank voles was inefficient (Beringue et al., 2008c).

The mechanisms underlying host interaction with TSE strains are not yet fully understood. Thus predicting from strain characteristics whether a strain will transmit to a specific host is not always possible. *In vitro* conversion assay systems can often give a good indication of efficient host and strain interactions suggesting that the conversion efficiency from PrP^C to PrP^{Sc} is a major determinant of transmissibility. However some discrepancies between *in vitro* systems and *in vivo* systems suggest that additional components influence susceptibility *in vivo* that are not always present in *in vitro*

systems eg the ability to convert PrP^C *in vitro* from apparently resistant species *in vivo* (Castilla et al., 2008a; Fernández-Borges et al., 2009; Vidal et al., 2010).

Conclusions:

- The TSE strain is a major determinant of the capacity for inter-species transmission.
- Transmissibility is dependent on the interaction between the specific agent and the host PrP.

2.2.3.3. The route of infection

Route of infection is also a factor that contributes to transmission efficiency both between and within species (Kimberlin and Walker, 1988; Mabbot and MacPherson, 2006). While experimental setups often focus on the most efficient route of transmission, the intracerebral route, natural infection usually occurs through peripheral routes such as the oral route, which have been demonstrated experimentally to be less efficient than the intracerebral route (Kimberlin and Walker, 1988), and consequently experimental interspecies transmission as observed after intra-cerebral/intra-peritoneal challenge might be difficult to interpret with regard to the transmission risk following exposure under natural conditions. Studies have demonstrated the intravenous route is an efficient route of infection, which raises issues concerning transmissions of TSEs through blood transfusion, blood products and pharmaceutical products (WHO, 2006, 2010).

Conclusions:

- The route of infection is also a factor that contributes to transmission efficiency both between and within species.

2.2.3.4. Outcomes of interspecies transmission

The cross-species transmission of TSE agents can lead to different outcomes, which may vary according to the strains involved and in an unpredictable manner in the present state of knowledge. The main outcomes observed include:

- i) Absence of observed transmission to the exposed host. The validity of this outcome is dependent on the number of exposed recipients, on the observation period in relation to the lifespan of the exposed host and on the number of serial passages within the host species. There is recent data that a species which was previously considered as resistant to experimental TSE transmission, the rabbit (Gibbs and Gajdusek, 1973), is also susceptible.
- ii) An asymptomatic disease (Hill et al., 2000; Race and Chesebro, 1998; Race et al., 2001) or a long-term “persistence” of the inoculated TSE agents in the exposed host (Dickinson et al., 1975; Nonno et al., 2006; Wadsworth et al., 2004).
- iii) A conservation of strain characteristics, as generally observed upon experimental transmission to transgenic mice expressing a PrP gene homologous to that present in the infecting source (Asante et al., 2002; Beringue et al., 2008b; Collinge et al., 1996; Le Dur et al., 2005; Scott et al., 1999). TSE agents may also maintain phenotypic traits upon transmission to hosts with heterologous PrP^C sequence, as exemplified by the remarkable ability of the BSE agent to retain its biological properties after experimental transmission to a strain typing panel despite intermediate passage in a range of different hosts with distinct PrP^C sequences (Bruce et al., 1994; Bruce et al., 1997).
- iv) The emergence of a new strain with unprecedented properties. One of the best studied example is the observation by Bessen and Marsh (1992a) that the serial transmission of one isolate of TME to hamsters resulted in two distinct disease phenotypes, termed Hyper and Drowsy (HY and DY,

respectively) because either hyperexcitability or drowsiness predominates at clinical stage. In addition, both incubation times and brain PrP^{res} molecular patterns differed in hamsters, indicating that two strains were isolated (Bessen and Marsh, 1994). DY but not HY retained pathogenicity for mink through at least four passages in hamsters, suggesting the possibility that it was the major if not the sole mink pathogen component in the original source (Bessen and Marsh, 1992b). Interspecies/heterologous transmission of cattle TSE isolates (Beringue et al., 2007; Capobianco et al., 2007) further illustrated the notion that the “new” emerging strain may have a phenotype converging towards a known one (convergence phenomenon).

More recently BSE agent stability after passage through sheep species has been investigated by a number of groups. The transmission features were compared with cattle BSE in mice transgenic for bovine PrP (Espinosa et al., 2009). While the molecular and pathological phenotypes were indistinguishable, sheep-passaged BSE induced a significantly shorter incubation period on the first but also subsequent passages in bovine transgenic mice, thus excluding different infectivity levels in cattle and sheep brains as a possible explanation. An increased pathogenicity of sheep-passaged BSE was also observed in mice transgenic for either porcine PrP (Espinosa et al., 2009) or human PrP (Padilla et al., In press; Plinston et al., 2010a), as well as in conventional RIII mice when compared with retrospective cattle BSE experiments (Gonzalez et al., 2007). These data raise the possibility that the BSE agent may gain virulence by passage in another species.

The recurrent dilemma with the emergence of a new strain is whether distinct (minor) strain components pre-existed in the original TSE isolate and observed clinical manifestation in the foreign host or whether a “truly new” variant TSE agent emerged upon confrontation to a foreign PrP sequence. Alternatively, two different TSE agents can show a phenotypic convergence in the new host.

v) Host adaptation

After crossing a transmission barrier and subsequent passages of a TSE agent in the new host, there can be a shortening of incubation times and a higher attack rate as the agent “adapts” to its new environment (Kimberlin et al., 1987).

vi) Conformational changes during interspecies transmission

While mutational events in agent-associated nucleic acid were originally cited as the cause of strain instability (Bruce and Dickinson, 1987), more recently, changes in the conformation of PrP^{Sc} have been shown to be associated with the acquisition of new strain properties (Peretz et al., 2002). To account for the phenomena of TSE agent transmission barriers, strain instability, heterogeneity, and adaptation in the context of PrP^{Sc} conformation, the conformational selection model postulates that only a subset of PrP^{Sc} conformations is compatible with each individual PrP primary structure (Collinge, 1999; Collinge and Clarke, 2007). By extension, this leads to the notion that TSE agents exist as quasispecies (Li et al., 2010), a model first applied to populations of a virus within its host (Eigen, 1996). Quasispecies acquire fitness when populations of prion conformers are subjected to selective pressure, for example during propagation in a host expressing a different PrP primary structure following interspecies transmission. From this perspective, host PrP^C primary structure influences the portfolio of thermodynamically preferred beta-pleated PrP conformations that are kinetically selected during propagation (Makarava and Baskakov, 2008; Rezaei et al., 2002).

Conclusions:

- The cross-species transmission of TSE agents can lead to different outcomes, which are, in the current state of knowledge, unpredictable.

Overall conclusions on the diversity of TSE agents, strain definition and transmission barrier:

- Multiple strains of TSE agents exist in each species, i.e. sheep, cattle, goats, cervids and humans.
- The full diversity of natural TSE agents is unknown.
- The potential for inter-species transmission is dependent on the TSE strain, the PrP gene of the host, the route of exposure and potentially other factors yet to be identified.
- Currently it is unpredictable whether a TSE agent will transmit to a new host. If transmission occurs, outcomes can range from no apparent disease to altered strain characteristics resulting in increased virulence in the new host.

3. Tools and methodologies

Tools that are available in TSEs are used to address a number of important issues:

- to diagnose and confirm the presence of a TSE;
- to identify presence or absence of infectivity and potentially measure infectious titres;
- to characterise the TSE agents infecting an animal or human;
- to assess the potential for TSE agents to cross a species barrier.

The methodologies used range from epidemiology to *in vivo* and *in vitro* methods. In most instances a combination of tools are required to address specific questions on the inter-species transmission potential of TSEs.

The following sections will assess the tools available and how each can provide information on the questions above.

3.1. Use of epidemiology of human and animal TSEs

3.1.1. Epidemiology and surveillance in humans

Collaboration on epidemiological surveillance of CJD was initiated in Europe in 1993, although some countries have systematic data on CJD from earlier years. At the onset of the EU study the following countries participated: France, Germany, Italy, the Netherlands, Slovakia and the UK. In 1997 additional countries joined the project (Austria, Australia, Canada, Spain and Switzerland) and in 1998, following the recommendation of the Council of the European Union, Belgium, Denmark, Finland, Greece, Portugal and Sweden joined, together with Iceland, Israel and Norway. In 2001 through EU funding the following countries became involved with the system: China, Czech Republic, Hungary and Poland. More recently, Latvia, Estonia and Slovenia have joined the surveillance network. The primary aim of the project was to harmonise methodologies for CJD surveillance, including case definitions, risk factor analysis and minimal monitoring datasets in order to allow comparability between countries and to identify new forms of CJD. In recent years additional countries have attended the annual meetings of the EURO-CJD group: Argentina, Japan, Mexico, South Korea and the USA.

The EURO-CJD group have established baseline information on the epidemiological characteristics of CJD of all type in Europe and have carried out extensive analysis of phenotypic characteristics, regional distribution by aetiology, risk factor analysis and basic epidemiological features, including mortality rates. In addition to research objectives relating to improved diagnosis, the group has made efforts to standardise specialist investigations and to obtain tissues and samples for diagnostic evaluation and research.

The surveillance system for TSE in humans is necessarily passive as there is no available specific *in vivo* diagnostic test and therefore the sensitivity of the system is limited both for the identification of known forms of human TSEs as well as potentially novel forms of human disease. An essential component of the system is to attempt to identify and verify all cases of CJD in participating countries through targeting of specific professional groups, mainly neurologists, neuropathologists and psychiatrists. Despite the variation in health care systems between countries, the mortality rates from participating countries are overall remarkably similar, suggesting relatively uniform case ascertainment. An important component of the EURO-CJD system is to share expertise between countries and, if necessary, laboratory and other investigations.

The availability of systematic epidemiological and phenotypic information on all forms of CJD in Europe is an essential prerequisite to the identification of novel forms of human disease, including any potentially linked to animal TSEs.

Conclusions:

- Systematic surveillance systems for all forms of CJD have been established in many countries, including the great majority of countries in the EU.
- The EU CJD surveillance system has been harmonised in relation to methods of case ascertainment, case definitions and risk factor analysis.
- A primary objective of this system is to identify novel forms of human TSEs.
- To date the only novel forms of human TSEs that have been identified in the EU are vCJD and a small number of cases associated with novel mutations of *PRNP*.
- The surveillance system for human TSEs is necessarily passive and therefore there may be some limitations in sensitivity.

3.1.2. Epidemiology and surveillance in animals

Knowledge about the spatial and temporal distribution of animal TSEs is based mostly on routinely collected data obtained through surveillance activities.

The peculiar characteristics of animal TSEs (heterogeneous clinical presentation, long incubation period ranging in years, extremely low incidence, no *ante mortem* tests available due to the lack of inflammatory or immune responses, and the accumulation of detectable PrP^{res} restricted essentially to the central nervous system (CNS)) have always made difficult the implementation of an effective monitoring and surveillance system.

Before 2001 for BSE, and 2002 for small ruminant TSEs, the epidemiological knowledge derived exclusively from passive surveillance, i.e. the mandatory reporting of clinically suspect animals and the confirmation of TSE.

After 2001 in cattle and 2002 in small ruminants, an EU wide active surveillance system has been implemented. Animals are identified within different risk streams e.g. as healthy animals at abattoir or fallen stock, and are submitted to the so called rapid tests for the presence of abnormal PrP in the brainstem. These activities, based on post mortem testing for the presence of abnormal PrP in the brainstem, involve a proportion of animals in the case of small ruminants (according to a sample-based scheme, which is applied also on BSE in non-EU countries), and all animals (exhaustive scheme) above a certain age in cattle.

The intensity of surveillance may vary by country, based on the sampling scheme applied and/or the proportion of animals tested by stream. The European scale of this monitoring system is clearly surpassing the minimal OIE requirements (OIE, 2010). It is based mostly on routinely collected data obtained through surveillance activities, which in the EU have been massive in the last 10 years and made available annually as reports on the monitoring and testing of ruminants for the presence of TSEs in the EU⁹.

⁹ Source: European Commission, available at http://ec.europa.eu/food/food/biosafety/tse_bse/monitoring_annual_reports_en.htm

Active surveillance revealed the poor performance of passive surveillance in terms of detection of TSEs both in cattle and in small ruminants (Ducrot et al., 2008), raising major concerns about the past epidemiological picture of TSEs. In particular it allowed the identification of BSE in many European countries thought as BSE-free, confirming the results of the Geographical BSE Risk assessment carried out by the EU (SSC, 2000). It also revealed the existence of unknown TSE forms like L-BSE and H-BSE (Biacabe et al., 2004; Casalone et al., 2004) in cattle, and Atypical scrapie/Nor98 in small ruminants (Benestad et al., 2003). These atypical TSE forms have now been identified in many countries outside the EU: H-BSE and/or L-BSE cases have been reported in North America and Japan, whereas Atypical scrapie/Nor98 cases were also detected in New Zealand and Australia, two countries considered so far to be free of TSE. However the sensitivity of the surveillance system in the detection of those atypical forms remains unknown. After nearly a decade, the EU active TSE monitoring system in cattle can be considered to have continuously provided a high quality picture of Classical BSE epidemics allowing deep and informative epidemiological analyses (Ducrot et al., 2010).

In small ruminants, the EU active surveillance resulted in an improved epidemiological description of the overall Classical scrapie occurrence and geographical distribution in the different Member States. Despite requirements foreseen by EU legislation in terms of sample size for the different risk categories and discrimination of BSE from other TSE agents, the sample-based scheme may allow a selective application of the testing both in terms of overall sample size, proportion of animals tested by risk stream (leading to different probability of detecting the disease) and geographical distribution: as a result, the occurrence and the geographical distribution of small ruminant TSEs may be biased. Moreover the absence of systematic characterization of all the TSE agents in the positive samples limits our comprehension of the TSEs complexity in the field. Finally, there still exists some concern on the operational performances of the monitoring system in identifying ruminants infected by distinct TSE agents as shown in particular for the atypical TSE agents (Casalone et al., 2004; EFSA Panel on Biological Hazards (BIOHAZ), 2010a; Fediaevsky et al., 2010; Fediaevsky et al., 2008).

Conclusions:

- The epidemiological knowledge of animal TSEs is mainly descriptive and relies on surveillance data.
- The peculiar features of TSE diseases (long incubation period, extremely low occurrence, and no *ante mortem* test available) make an effective monitoring and surveillance system difficult to run. The intensity of surveillance is variable, as different sampling schemes are applied internationally.
- Before 2001/2002, TSE monitoring in animals relied on passive surveillance, which is of limited sensitivity for assessing the occurrence and the geographical distribution of TSEs in animal populations.
- Systematic monitoring of cattle after 2001 by means of active surveillance has provided a more complete picture of the occurrence of Classical BSE in cattle in the EU.
- Sample-based monitoring of TSEs in small ruminants after 2002 by means of active surveillance provided valuable data on the occurrence of TSEs in these species, but the obtained information has some limitations with regard to the geographical distribution and prevalence.
- In small ruminants, the TSE surveillance system does not allow to differentiate between TSE agents causing Classical scrapie.

- The active screening has allowed the identification of three new forms of animal TSEs (L-BSE, H-BSE, Atypical scrapie). However, the information obtained has major limitations due to the unknown sensitivity of the monitoring system for these TSEs.

3.1.3. Comparison of human and animal epidemiology

For animal TSEs, including recently identified atypical animal TSEs, the examination of a possible epidemiological link between animal and human cases depends on knowledge of the distribution of cases temporally and geographically. This methodology provided evidence for establishing the causal link between BSE and vCJD.

Search for the aetiology of sCJD has been the subject of many studies over past decades, including both observational and analytic epidemiological research. Some of the older descriptive studies, which were based on historical aggregated data (Chatelain et al., 1981; Masters et al., 1979), did not provide systematic information on CJD and therefore cannot be considered to be informative on aetiology. Case-control studies in sCJD have failed to identify any consistent risk factor for the development of disease (Wientjens et al., 1996), and, although some of these studies have identified weak associations between sCJD occurrence and a number of putative risk factors, they have often been contradictory. These studies may be compromised by limited power and potential bias in relation to selection and interview in control groups (Barash et al., 2008). In conclusion, no major environmental sources of infection for sCJD have been identified, but the capability of these studies to exclude minor environmental risk factors is limited.

sCJD appears to have a relatively constant mortality rate in systematic studies, including studies from many countries in Europe (Ladogana et al., 2005) and elsewhere. Overall, cases of sCJD appear to occur randomly in time and space. Although occasional clusters of cases have been identified (Arakawa et al., 1991; d'Aignaux et al., 2002; Linsell et al., 2004), raising the possibility of an environmental source of infection, the significance of these findings is uncertain (Klug et al., 2009). These observations have led to the plausible view that sporadic CJD is not environmentally acquired and is most likely caused by somatic mutation or by the spontaneous conversion of the normal form of PrP to the disease associated form with subsequent replication and disease (Prusiner, 2001).

This hypothesis is not consistent with all the known characteristics of sCJD, for example the presently available data on age-specific incidence that declines in the elderly. From an epidemiological perspective, the evidence in relation to sCJD cannot be regarded as definitive, and the possibility that a small proportion of cases are zoonotic cannot be excluded. However, evidence of a mismatch between potential human exposure to scrapie and the mortality rates for sCJD, e.g. in Australia, New Zealand (Will et al., 1996), Iceland (Georgsson et al., 2008) and Cyprus (Papacostas et al., 2008) argue against this possibility. Furthermore, genetic forms of CJD are thought to be due to an increased likelihood of spontaneous misfolding of normal PrP as a result of the associated mutations of the prion protein gene (Prusiner, 2001), and this would be consistent with the hypothesis that sCJD has a similar mechanism. Notably the age at onset of disease in sCJD is relatively delayed in comparison to genetic forms of CJD, which would be consistent with the hypothesis of increased instability in prion protein in association with *PRNP* mutations, although the proposal is that both sporadic and genetic CJD share an identical basic causal mechanism.

The epidemiological evidence in relation to sCJD has accumulated over decades of research. This long-standing evidence contrasts with the relative freshness of data on animal TSEs and in particular newly identified atypical TSEs.

There is systematic and validated data available on human TSE diseases in most EU Member States through the EURO-CJD system. For some countries data on CJD has been accrued since the early 1990s and for the majority of other countries since the late 1990s. A minority of countries have historical data on CJD from previous periods. Importantly, mortality rates for sCJD are relatively

uniform between countries, suggesting comparable levels of case ascertainment and that any risk factors for human TSE disease are evenly distributed, with the exception of historical exposure to BSE. No new form of CJD other than vCJD has been identified, as yet, through the EURO-CJD system.

Data related to past occurrence of animal TSEs considered in their diversity remain uncertain. Indeed before the implementation of the active surveillance program the information collected through the passive surveillance system should be considered with caution. Similarly, both Atypical scrapie and BSE are mainly identified through the active testing programme for animal TSEs in abattoirs, whose sensitivity for these TSE agents is unknown.

Comparisons of data between countries are compromised by differences in historical and current testing policies, including the ages of tested cohorts, and the true incidence and distribution of atypical animal TSEs are unknown. Furthermore, whether these diseases are newly occurring or have been prevalent in the long term and only recently identified is also unknown. The potential incubation period of TSEs in humans (up to several decades) and the relative short period (eight years) over which the active surveillance programs have been established in farmed ruminants compromise their use in the identification of a possible association between TSEs in animals and humans (other than vCJD). These uncertainties indicate that currently even a rough comparison of the epidemiological patterns between human and animal TSE diseases (other than Classical BSE) is unlikely to be informative.

Nonetheless, an important imperative is to continue to carry out systematic surveillance of human TSE disease in order to identify any newly emerging disease phenotype and to critically evaluate any possible epidemiological links between animal and human TSEs. Further delineation of the incidence of atypical animal TSEs will be a critical component of an assessment of any such link as a true differential regional incidence of these conditions may allow a comparison with emerging findings from the human surveillance programme.

Conclusions:

- Overall, cases of sCJD appear to occur randomly in time and space.
- There is currently no evidence of environmental sources of infection in sCJD.
- The epidemiological evidence in relation to sCJD cannot be regarded as definitive, and the possibility that a small proportion of cases are zoonotic cannot be excluded.
- Except for the Classical BSE agent, there is limited epidemiological information on the prevalence and distribution of individual ruminant TSE agents.
- These uncertainties indicate that even a rough comparison of the present epidemiological patterns of human TSEs and animal TSEs other than Classical BSE is unlikely to be informative.
- To reduce these uncertainties, it is an imperative to continue to carry out systematic surveillance of human TSEs, and to continue and improve the surveillance of animal TSEs.

3.2. *In vivo* methodologies

3.2.1. Neuropathology/PrP deposition patterns

Neuropathological studies of both human and animal TSEs have demonstrated the occurrence of relative similar lesions in the CNS. This concerns both classical histopathological features such as spongiform change, neuronal loss and gliosis, and the deposition of disease-associated PrP.

In rodent models, after experimental transmission of TSE isolates, the semi-quantitative scoring of vacuolar changes in standardized brain regions (named “lesion profiling”) of diseased animals, is one of the keystone features used for establishing the phenotype of the involved agent (strain typing) (Bruce et al., 1991; Fraser and Dickinson, 1968). This approach is complemented by assessment of astrogliosis and microgliosis and by the characterization of the PrP deposition pattern both at tissue level (Histo Blot – PET blot) (Beringue et al., 2008c; Taraboulos et al., 1992; Wemheuer et al., 2009a) or microscopic level (immunohistochemistry) (Beck et al., 2010b).

“Lesion profiling” for characterizing TSE agents in rodent models is supported by a large body of data. In particular, lesion profiling in RIII mice was one of the key features for demonstrating that BSE and vCJD were caused by the same TSE agent (Bruce et al., 1997). However the approach also needs to consider several issues that include the necessity of sub-passage in most cases, the number of animals necessary for reliable profiling, and the possibility of a divergent profile arising from a single isolate (Thackray et al., 2008). Recently it has also been reported that lesion profiling in RIII could be an insufficient basis on its own, to distinguish BSE from some scrapie isolates (Beck et al., 2010a). Host properties can dramatically affect lesion profiles, independent of PrP genotype. Different mouse strains have different lesion profiles and this has obvious implications for attempts to perform lesion profiles or other forms of quantitative, comparative pathology in outbred species such as sheep or humans. It should be noted that lesion profiling is never used in isolation but as one of a number of tools. Lesion profiling alone is not sufficient as two different strains can have the same lesion profile but will be discriminated on other criteria such as incubation time.

In humans, neuropathological examination still remains one of the most important tools for a definite diagnosis of TSE and for the recognition of novel phenotypes. Sporadic CJD is defined to feature spongiform changes in cerebral and/or cerebellar cortex and/or subcortical grey matter; and/or as encephalopathy with PrP immunoreactivity (diffuse synaptic and/or patchy/perivacuolar and/or plaque type) (Budka, 2003). Despite these basic features, human TSEs have highly variable neuropathology as part of a wide range of phenotypic variation, reflecting not only the type of disease (or “strain”), but also other factors such as the duration of disease, and may be significantly affected by the route of infection (Langevin et al., 2011). However, the elucidation of the molecular basis of human TSEs allowed recognition of specific factors that correlate with neuropathology. In sCJD, distinct lesion profiles and PrP deposition patterns have been described as specifically corresponding to the *PRNP* codon 129 genotype and PrP^{res} type (Parchi et al., 1999). In vCJD, brain pathology is relatively uniform in the *PRNP* M129M patients so far studied (Ironside et al., 2000). In genetic human TSEs, the codon 129 polymorphism has a phenotypic influence, as in non-genetic forms, but additional factors might be considered as background for phenotypic variability (Kovacs et al., 2002). One particular example is GSS where the same genetic constellation (P102L M129M) has two distinct phenotypes (Hainfellner et al., 1995).

In cattle affected by natural Classical BSE, a relatively “stable” distribution and scoring (intensity) of the vacuolar changes and abnormal PrP distribution pattern were reported (Scott et al., 1990; Simmons et al., 1996; Wells et al., 1991). In BSE inoculated and scrapie inoculated cattle (intra-cerebral) (Konold et al., 2006) both these features were clearly different. Similarly the PrP distribution pattern reported in Atypical L-BSE (on the basis of a limited number of natural L-BSE cases) and Classical BSE cases seems different (Casalone et al., 2004). Together these data support the contention that neuropathological features can be useful for assessing involvement by the same or different agents in TSE affected cattle.

In small ruminants the situation appears more complex. Neuropathological features were reported as a relatively straightforward approach to distinguish Atypical scrapie from other TSE agents (Mazza et al., 2010; Moore et al., 2008; Simmons et al., 2010a; Wemheuer et al., 2009b) and Classical scrapie.

In field Classical scrapie cases, differences were reported in the distribution of CNS lesions (Gonzalez et al., 2010). However, for the same TSE agent, the vacuolar changes distribution and

intensity seem to vary considerably according to considered individuals, which preclude to consider lesion profiling as a solid basis for identification of Classical scrapie agents (Begara-McGorum et al., 2002; Gonzalez et al., 2010; Jeffrey and Gonzalez, 2007; Ligios et al., 2002).

Neuropathological features common to both human and animal TSEs mainly comprise spongiform changes/vacuolation and PrP immunoreactivity/accumulation of PrP^{res}. Neuropathological features that differ between human and animal TSEs comprise neuronal loss that is conspicuous in human, but not in natural animal TSEs (role of disease duration?); intraneuronal vacuoles that are conspicuous in many natural animal TSEs, but not in human TSEs; regional vulnerability that shows more diffuse (cerebral and/or cerebellar cortex and/or subcortical grey matter) involvement in humans and Atypical BSE/scrapie, vs. predominantly brainstem pathology in Classical BSE and scrapie (role of spontaneous development vs. centripetal acquisition of the infection?).

Lymphoid system involvement is prominent in Classical scrapie, CWD and vCJD, but not in other human TSEs, Atypical scrapie, Classical BSE and Atypical BSE.

Involvement of the lymphoid system in human TSEs is uniform in vCJD (Ironsides et al., 2000), whereas its minor occurrence in sCJD seems to mainly depend upon the length of disease (Glatzel et al., 2003).

The only known human TSE acquired from an animal source, vCJD, demonstrates neuropathological features that greatly differ from the neuropathology by the donor strain in the original host (BSE in cattle) (Ironsides et al., 2000). On the other hand, there might be a striking similarity of neuropathologies in animal and human brains despite distinct strains and disease types, such as the striking, vCJD-like presence of abundant florid plaques in CWD of captive mule deer (Luberski et al., 2001). More recently, similarities in the PrP deposition pattern detected by the PET blot method were described in sCJD type 1 and the so-called Atypical/Nor98 scrapie on the one hand, and sCJD type 2 and Classical scrapie on the other; this phenomenon was interpreted to suggest grouping of TSE diseases in which common TSE agent “types”, not strains, are the major determinant for TSE disease forms (Wemheuer et al., 2009a).

Conclusions:

- In humans, neuropathological examination remains one of the most important tools for a definite diagnosis of TSEs and for the recognition of novel phenotypes.
- Brain pathology (histopathology and PrP deposition pattern) is relatively consistent according to the affected host and the involved TSE agents. Even with the same strain, however, high individual variability is seen in natural Classical scrapie and some human TSEs which are likely to be due to differences in the genetic background of the host. Common pathology (i.e. common targeting) in different hosts does not necessarily imply strain similarities.
- The distribution of neuropathology can be significantly influenced by the route of exposure in acquired TSEs.
- Lesion profiling in defined (ideally inbred) hosts is an important tool in strain typing.
- In human TSEs, neuropathology is part of the considerable phenotypic variation and mainly but not exclusively reflects distinct molecular characteristics of the host *PRNP* genotype including codon 129 and PrP^{res} type.
- In human TSEs, the extent of the involvement of the lymphoid system may be strain-specific, but may also depend on additional factors (e.g. disease duration).

- The neuropathology of vCJD greatly differs from that of BSE in cattle: the same TSE strain can cause distinct neuropathologies in distinct hosts.
- The neuropathology of some human TSEs may resemble that of some animal TSEs: different TSE strains may cause similar neuropathologies in different hosts.
- Neuropathology and PrP deposition patterns in isolation do not allow conclusions on the source of a TSE strain across a species barrier.
- The clinico-pathological phenotype in a human disease that might be caused by atypical animal TSEs cannot be predicted.

3.2.2. Transmission experiments

3.2.2.1. Wild type mouse panels

Historically, strain typing protocols in wild type mice based on intracerebral inoculation with brain extract and determining neuropathological characteristics and the incubation period to clinical disease have been used widely for many laboratory isolates.

Different laboratory TSE strains tested in the same mouse strain can give markedly different incubation periods. If experimental conditions are constant the incubation period for a particular strain in a group of genetically uniform mice is highly predictable, also highly repeatable for different groups of genetically uniform mice injected with the equivalent doses of the same TSE strain. The incubation period for strains is also influenced by genetic factors in the mouse. Two alleles of the murine PrP gene have been recognised (designated a and b), encoding proteins that differ at codons 108 (leucine for genotype a mice, phenylalanine for genotype b mice) and 189 (threonine for genotype a mice, valine for genotype b mice). When mice are inoculated with a single laboratory TSE strain, the rate of progression of disease varies according to the genotype of the mouse, making differences of hundreds of days to the incubation period. Each TSE strain gives a characteristic and highly reproducible pattern of incubation periods in the 3 possible mouse genotype combinations (homozygote a, homozygote b and ab heterozygote). On initial transmission of TSE to mice from a different species, the incubation period is usually long, but on subsequent mouse to mouse transmissions (passages), the incubation period shortens and, after further passages, stabilises. The incubation period and neuropathological characteristics become stable indefinitely on further mouse to mouse passage as long as the conditions of passage, especially the host mouse PrP genotype, remain constant.

Laboratory TSE strains show differences in the type, severity and distribution of pathological changes they produce in the brains of infected mice (Bruce, 2003). In histological sections a semiquantitative method of strain discrimination is employed which scores the severity of vacuolation in coded sections in nine grey matter and three white matter brain areas to construct a lesion profile, characteristic for each combination of TSE strain and mouse genotype (Fraser and Dickinson, 1968). Immunostaining of brain sections with PrP-specific antisera can reveal accumulations of PrP in the form of diffuse deposits in vacuolated areas and, more focally, as amyloid plaques. Some murine TSE strains specifically target PrP pathology to particular groups of neurons, leaving the surrounding brain unaffected, whereas others produce a more generalised PrP distribution still within particular brain areas. Some strains produce many amyloid plaques while others produce few or none.

A list of wild type mice used in the TSE field is provided in Appendix A, Table 1.

3.2.2.2. Transgenic mouse models

Minimal amino acid divergences may have a major impact on the transmission efficiency. Early studies suggested that the cross-species barrier resides essentially in PrP primary structure differences between the host and donor species. In seminal experiments, Scott et al. (1989) abrogated the recognized resistance of the mouse to hamster scrapie by expressing hamster PrP^C in transgenic mice. This apparent lack of species barrier after homotypic transmission, i.e. when the host expresses a PrP gene identical to that of the infecting species, has led to the development of a long list of mice transgenic for sheep, bovine, human, cervid and mink PrP (See Appendix A, Tables 2 and 3). A variety of different transgenic mice expressing chimeric versions of PrP in which specific regions of mouse PrP primary structure were replaced by the corresponding elements from human (Telling et al., 1994; Telling et al., 1995), sheep (Gombojav et al., 2003; Kupfer et al., 2007) and bovine PrP (Kupfer et al., 2007; Scott et al., 1997) have been created. The TSE agent/strain typing methods developed with these lines are essentially similar to those used in wild-type mice (See above).

Most of such lines were obtained by standard transgenic approaches, and have an endogenous PrP null (PrP0/0) background, in order to avoid any interfering effect of the resident murine PrP gene. An inverse correlation between the length of survival time and expression level of the transgene in the brain has been noticed in mice transgenic for mouse, hamster, sheep and bovine PrP.

Some transgenic lines have been established by a gene replacement method (See Appendix A, Table 2). This technique generates transgenic mice by homologous recombination after transfecting murine embryonic stem cells with the gene of interest and inserting by homologous recombination at the defined target site in the mouse genome ensuring the gene is under the same regulating elements as the wild type gene. Most gene targeted mice are also inbred lines (Ola 129 background), so the only difference between each line is the precise alteration introduced into the PrP gene.

In both cases, PrP sequence identity between the transgenic host and donor are usually associated with a higher transmission rate as compared to wild-type mice.

Increased PrP expression can shorten incubation time. Several studies indicated no alteration of susceptibility linked to PrP overexpression (Buschmann and Groschup, 2005; Peretz et al., 2006). However, this point is still debated and further research is needed. The shortest incubation times tend to be in transgenic mice in which PrP^C is over-expressed due to a multiple transgene copy insertion after pronucleus microinjection. It should be noted however that incubation times in low level expression models are not necessarily long but result from a combination of host and agent (Bishop et al., 2010).

The use of TSE transmission models might be limited by the life span of the recipient host, which might be exceeded by the incubation period. Long incubation times, particularly during interspecies transmission, may therefore simply be missed when the recipient host dies naturally before the end of the incubation time, although pathological examination, PrP^{Sc} detection and secondary passage may identify infected animals. These problems can be partially overcome by transgenic mice over expressing PrP^C with a considerable reduction of the incubation time.

3.2.2.3. Primate models

Historically, first experimental evidences of the transmissibility of human TSE diseases were obtained in primate models. Kuru was the first human spongiform encephalopathy to be demonstrated as transmissible by intracerebral inoculation to chimpanzee (Gajdusek et al., 1966), to different species of new-world monkeys (Gajdusek and Gibbs, 1971) and then to rhesus macaque (Gajdusek and Gibbs, 1972). In parallel, the infectious aspect of sCJD was initially exhibited using the same primate models (Gajdusek and Gibbs, 1972; Gibbs et al., 1968; Zlotnik et al., 1974), and consecutively demonstrated

in other various species of old-world monkeys including cercopithecus and baboons (Brown et al., 1994; Goudsmit et al., 1980).

Clinical and pathological similarities between those experimentally induced diseases in primates on one hand, and corresponding natural diseases in human on the other hand have been recurrently described (Beck et al., 1966, 1969a; Brown et al., 1994; Williams et al., 2007). Conversely, experimental attempts to transmit both of those human diseases to other conventional (i.e. non transgenic) animal models (ruminants, carnivores or rodents) failed (Gibbs and Gajdusek, 1973): despite their long incubation periods and the ethical dilemma of their use in research, primate models were therefore rapidly considered as the utmost relevant experimental models for risk assessment of TSE agents for human health. They notably allowed not only the first demonstration of the presence of infectivity in suspect samples in the context of iatrogenic outbreak of CJD linked to human growth hormone therapy (Gibbs et al., 1993), but also the transmissibility of TSE agents through contaminated surfaces of medical devices (Gibbs et al., 1994).

According to their proximity to humans (non-human primates share high level of genome homology with humans, and they notably show 96 to 99 % homology to the human sequence of PrP amino-acids (Cervenakova et al., 1994; Schätzl et al., 1995)) primates are then considered as the most relevant conventional animal model for risk assessment of TSE diseases for human health. Phylogenetically speaking, Chimpanzee (*pan troglodytes*) is the closest model to human, but its introduction in research protocols is now severely controlled and limited. Among the different remaining animal models, old world monkeys including macaques are evolutionary closer to human (Hayasaka et al., 1988), but their use is controlled and expensive. Conversely, squirrel monkeys (new world monkeys) and mouse-lemurs (prosimians) are easier to house and handle but are farther from humans. Those phylogenetic distances (See Table 2 below) may influence the pertinence of those models for assessing zoonotic risks of animal TSEs: for example, squirrel monkeys are highly susceptible to CWD (88% of transmission after 33-53 months of incubation) (Marsh et al., 2005) while macaques seem more resistant (no transmission 70 months post-inoculation) (Race et al., 2009b). However it should be noted that host PrP sequence alone does not determine susceptibility but a combination of strain of agent and host as seen in other species.

Table 2: Phylogenetic classification of primates.

Order	Sub-Order	Infra-Order	Parv-Order	Super-Family	Family	Species	
Primates	Strepsirrhini (Prosimians)	Lemuriformes			Cheirogaleidae (32 species)	Mouse-lemurs	
					Lemuridae (22 species)	Lemurs	
		Lorisiformes		Lorisidae (9 species)	Lorises		
	Haplorrhini (tarsiers, monkeys and apes)	Tarsiiformes				Tarsiidae (9 species)	Tarsiers
						Simiiformes	Platyrrhini (NewWorld Monkeys) (139 species)
		Cebidae	Squirrel monkeys				
		Atelidae	Spider monkey				
		Catarrhini (Old World Monkeys)		Cercopithecoidea	Cercopithecidae (135 species)	Macaques Baboon	
					Hominoidea	Hylobatidae (13 species)	Gibbon
						Hominidea (7 species)	Chimpanzee Human

When BSE emerged, raising the question of its transmission to humans, a second set of experimental transmissions to primates was set up. They demonstrated the transmission of this animal TSE strain to different primate species, including marmoset (Baker et al., 1993), cynomolgus macaque (Lasmez et al., 1996), lemurs (Bons et al., 1999) and squirrel monkeys (Williams et al., 2007).

A transmission barrier, even slight, exists between those expensive primate models and humans, while it is inexistent in humanized transgenic mice, suggesting *a priori* that TSE features induced by a specific prion strain in those latter models may be more relevant of those observed in humans. Nevertheless, lifespan of primates (especially old-world monkeys) may exceed several decades, allowing the expression of TSEs with long incubation periods in those animals contrarily to mice. Moreover, according to their similarities in terms of physiology and anatomy, primates are optimal models to assess risks of real transmission to human in natural conditions (e.g. oral exposure, peripheral transmission through blood).

3.2.2.4. Other species models

Historically, in the absence of knowledge on the nature of the transmissible agents responsible for TSEs, bioassays were carried out in various animal models.

Gibbs and Gajdusek (1972) have provided a comprehensive view of animal and human TSEs host range¹⁰. Among these are hamsters, which have proved very useful in TSE physiopathology and potential of adaptation following cross-species transmission events. However these animals did not appear to be susceptible to *bona fide* human TSEs cases, thus precluding any phenotypical comparison with animal TSEs.

Similarly, other laboratory animals such as rats, guinea pigs, rabbits, hens, ducks, and dogs appeared (on the basis of a limited data set) unsusceptible to either Kuru or CJD isolates (Gibbs and Gajdusek, 1973). However, more recently *in vitro* data suggested that some of those animals may be susceptible to TSEs in the experimental setting (Castilla et al., 2010).

In marked contrast, sporadic and familial CJD cases appear to transmit with little or no transmission barrier in bank voles (*Myodes glareolus* (Nonno et al., 2006)). As certain sheep scrapie agents appear to propagate in these animals, it may be feasible to use this newly developed model to address potential phenotypic similarities between human and animals TSEs. However at this stage, transmission data produced in this model remains too limited to allow drawing any definitive conclusion.

In TSE infectiology, the experimental use of ruminant models is generally limited to within-species pathogenesis studies and to the potential of interspecies transmission of TSE agents, such as those suspected/feared to transmit from ruminants to small ruminants (or inversely). For example, sheep and goats are fairly susceptible to cattle BSE, by intracerebral, intraperitoneal or oral routes of infection. Theoretically, CJD isolates could be experimentally transmitted to ruminants to address a potential link with ruminant TSE agents. However, such experiments have rarely been attempted, most likely for practical reasons. Nonetheless, at the beginning of the 80s, Hadlow et al. (1980) have inoculated goats with brain homogenate from sCJD-affected patients by intracerebral route. Some of the inoculated animals developed a TSE with “scrapie-like” neuropathology, indicating that the transmission barrier in goats is not absolute for human TSE agents.

¹⁰ It should be noted that the “scrapie” isolate used in these experiments was not a field scrapie isolate but had been serially passaged in laboratory rodents (Gibbs and Gajdusek, 1972).

Overall conclusions on transmission experiments:

- Two types of transmission experiments can be used to assess the zoonotic properties of animal TSE agents:
 - The inoculation of animal TSE agents to a model relevant for the human transmission barrier in order to estimate the potential of such agent to induce infection/disease in human.
 - The comparison of disease phenotype of human and animal TSE isolates after their propagation in the same animal models (conventional or transgenic rodent or other models), so as to identify the involvement of a potentially common TSE agent.
- These approaches are complementary. They provide different information, and cannot substitute to each other.
- Human PrP transgenic mice and primates are the most relevant models for investigating the human transmission barrier. To which extent such models are informative for measuring the zoonotic potential of an animal TSE under field exposure conditions is unknown. Because the Classical BSE agent is known to be zoonotic and to propagate with more or less efficacy in these models, the latter might be used as a benchmark to evaluate the zoonotic potential of other animal TSE agents.
- The significance of the experiments in human transmission barrier models is directly dependant on its design: the inoculation of a limited number of animals or an insufficient observation period could lead to a false negative result.
- Because of the potential impact of interspecies transmission on TSE agent properties (e.g. “convergence” phenomenon), the results of comparative study of human and animals TSE isolates after their transmission into a common host species should be considered with caution. A way to circumvent this would be to compare their behavior in several experimental models, if possible.

3.3. *In vitro* methodologies

3.3.1. Biochemical methodologies

The exact nature of TSE infectious agents remains unknown. However, to date it has been established that its main component is a host encoded protein (PrP^C) which is converted during the pathological process into an abnormal isoform (PrP^{Sc}). This abnormal isoform accumulates in tissues of affected individual (mainly the CNS, and in some cases other peripheral tissues like lymphoid tissues).

Characterization of PrP^{Sc} in laboratory animal models in the early 90's indicated that in particular host species, specific biochemical properties (like solubility in N-laurylsarcosyl or SDS PAGE WB pattern) (Bessen and Marsh, 1992a, 1994) can be associated with biologically different TSE agents. *In vitro* PrP^C to PrP^{Sc} conversion experiment resulted in the generation of new PrP^{Sc} molecules harbouring similar biochemical properties as PrP^{Sc} used as seeding particles (Bessen et al., 1995; Hill et al., 1997; Vorberg and Priola, 2002).

Those findings progressively lead to the statement that the PrP^{Sc} biochemical phenotype SDS PAGE WB pattern, PK degradation persistence (Simon et al., 2008), and resistance to denaturation (Safar et al., 2002) could be used to identify particular TSE agents.

Substantial progress has been made in recent years in the molecular characterisation of TSEs in ruminants and in humans, notably following studies aiming to differentiate BSE from other TSEs, and

in investigating the link between BSE and vCJD (Collinge et al., 1996). It indeed appeared that the BSE agent had highly stable features in cattle and that some of these particular PrP^{Sc} properties were also observed after passage of BSE in hosts from other species (Bencsik and Baron, 2007).

Moreover, tests were developed on the basis of biochemical abnormal PrP properties aimed at discriminating the BSE agent from other TSE agents, in particular in small ruminants (Stack et al., 2009). However, the specificity of these tools seems imperfect (Baron and Biacabe, 2007; Simon et al., 2008), and their sensitivity is still subject to debate (EFSA, 2007a, 2008b). These limitations have been recently substantiated by a study carried out in sheep experimentally challenged with a mixture of Classical BSE and Classical scrapie agents. In these animals, while a discriminatory assay applied to the brainstem resulted in the unequivocal identification of Classical scrapie, presence of the BSE agent was clearly identified (on the basis of biochemical properties) in the peripheral tissues of the affected individual (Lantier et al., 2008).

Analyses of brain PrP^{res} from natural scrapie cases have also revealed distinct molecular features, consistent with the existence of different strains. Three major PrP^{res} types have been identified, with unglycosylated forms migrating at ~21 kDa as in SSBP/1, at ~19 kDa as in CH1641 or BSE-like isolates, or showing the additional presence of truncated fragments at ~10–12 kDa visible in Atypical scrapie isolates (Hope et al., 1999; Lezmi et al., 2004; Race et al., 2002; Zanusso et al., 2003). Exactly how these PrP^{res} types correlate to TSE strains is not yet known.

PrP^{res} electrophoretic profiles are relatively homogeneous among cervids, both in terms of protease-resistant fragments size (~21 kDa for unglycosylated PrP^{res}) and glycopattern (Browning et al., 2004; Race et al., 2002; Xie et al., 2006). An alternative 19kDa pattern has been observed in diseased Rocky Mountain elk with leucine at codon 132 (O'Rourke et al., 2007). This was associated with a longer incubation than for animals carrying the 132-methionine allele. Whether this finding reflects different strains or the propagation of the same strain on two distinct PrP genotypes is unclear. Transgenic mice expressing elk PrP with L at 132 are also markedly resistant to CWD (Green et al., 2008b).

Similarly, in sCJD, two major PrP^{res} types have been described by Western Blot: in type 1 PrP^{res} the unglycosylated fragment is 21 kDa, while in type 2 the apparent molecular weight of this unglycosylated fragment is 19 kDa (Parchi et al., 1996). Protein N-terminal sequencing revealed that the type 2 isoform derives from preferential cleavage of the protein during PK digestion at amino acid 97, while in type 1 preferential cleavage occurs at amino acid 82 (Parchi et al., 2000). sCJD cases can be subclassified according to the PrP^{res} isoform and the *PRNP* codon 129 methionine (M)/valine (V) polymorphism, resulting in 6 major subtypes: MM1, MM2, MV1, MV2, VV1 and VV2. Interestingly, these subtypes appear to carry distinct pathological and clinical features (Parchi et al., 1996; Parchi et al., 1999) and it has been proposed that type 1 and type 2 isoforms in sCJD might correspond to different TSE agent strains. However, the description of PrP^{res} isoforms which appear to be distinct from type 1 and type 2, and the increasing number of reports describing the coexistence of type 1 and type 2 PrP^{res} in different areas or the same area in the brain from a single sCJD patient, calls into questions the subclassification system described above in sCJD (Dickson and Brown, 1999; Polymenidou et al., 2005; Puoti et al., 1999; Schoch et al., 2006).

Some authors compared abnormal PrP biochemical properties of animal and human TSEs isolates and noticed some molecular similarities. Such observations have been reported in both atypical forms of BSE (Biacabe et al., 2007; Casalone et al., 2004) and in Nor98 or Atypical scrapie in small ruminants (Arsac et al., 2007; Everest et al., 2006; Klingeborn et al., 2006; Saunders et al., 2006). However, these data could only suggest some similarities in the molecular mechanisms of these TSE diseases, which can lead to shared phenotypic features. Indeed, the PrP^{Sc} signature associated to a particular TSE agent (strain) depends on the agent, the host PrP sequence and the cell/tissue environment where the protein accumulates. This was clearly demonstrated by studies carried out with the Classical BSE agent that acquires slightly or radically different PrP^{Sc} phenotypes according to the host where it is

propagated; for instance Classical BSE in sheep transmitted to porcine animals has no common PrP^{Sc} features with Classical BSE in cattle (Castilla et al., 2004; Espinosa et al., 2007a).

Conclusions:

- The biochemical properties of human and animal PrP^{Sc} can be used as part of a classification system for disease phenotypes within a particular species. However, similar or even identical PrP^{Sc} biochemical signatures in different cases within the same or in different species are not interpretable in isolation as proof of infection with the same TSE agent.
- The PrP^{Sc} signature associated to a particular TSE agent depends on the agent, the host PrP sequence and the cell/tissue environment where the protein accumulates.

3.3.2. Conversion assays

3.3.2.1. Cell-free conversion

In 1994, the cell-free formation of a protease resistant prion protein was published by Kocisko et al. (1994). At that time the protein only hypothesis was far from being established, so these experiments were key for moving forward in this direction. The conversion mechanism and the relationship of PrP^{Sc} formation to TSE agent replication still remain unclear. Caughey (2000) reported the conversion of PrP^C to protease-resistant forms similar to PrP^{Sc} in a cell-free system composed of substantially purified constituents. This conversion was selective and required the presence of preexisting PrP^{Sc}, providing direct evidence that PrP^{Sc} derived from specific PrP^C-PrP^{Sc} interactions.

For cell-free conversion, PrP^C is produced in cells in a radiolabeled form and separated by immunoprecipitation. The PrP^{Sc} acting as seed is purified by conventional methods and both highly purified components are mixed and incubated in the absence of any other component. To evaluate the amplification of the infectious material, radioactivity of the new PK resistant PrP is measured. The efficiency of the conversion occurring *in vitro* is very low. A series of critical experiments followed this proof of concept. One of the most interesting ones was to use it as a model for the scrapie species barrier for mouse, hamster, and chimeric PrP molecules (Kocisko et al., 1995). Combinations of hamster PrP^C with hamster PrP^{Sc} and mouse PrP^C with mouse PrP^{Sc} was observed whereas non-homologous conversion reactions were, if at all, hardly efficient. Glycosylation of the PrP^C precursors was not required for species specificity in the conversion reaction. The same group studied the molecular assessment of the potential transmissibility of BSE and scrapie to humans using the same methodology. They concluded that there was a correlation between *in vitro* conversion efficiencies and *in vivo* transmissibilities of BSE, sheep scrapie and CJD suggesting a similar low possibility of transmission to humans of scrapie and BSE (Raymond et al., 1997). Other cell-free conversion studies showed the conversion of radiolabelled cervid PrP^C by CWD derived PrP^{Sc} in deer and elk *in vitro*. However, human or bovine PrP^C was much less efficient indicating a strain specific transmission barrier (Raymond et al., 2000). Other assays replaced the PrP^C substrate purified from mammalian cells with prion protein generated by baculovirus-infected insect cells (Iniguez et al., 2000) or used bacterial PrP^C (Kirby et al., 2003). Radiolabelled PrP^C was eventually replaced by L42 epitope (W144Y) tagged PrP^C expressed in *E. coli* which was used under semi-native cell-free conversions conditions (Eiden et al., 2006a). Using this assay Kupfer et al. (2007) could demonstrate the influence of single amino acid substitutions in murine/bovine PrP^C chimeras in the conversion by two different TSE strains. These results suggest that the effect of single amino acid substitutions and strain specificities observed *in vivo* may be encoded by the intrinsic properties of PrP^C and PrP^{Sc} (Eiden et al., 2006b).

The cell-free conversion technique was the gate for new experiments trying to study other transmission barriers, but also was important for developing PMCA.

3.3.2.2. PMCA

Protein Misfolding Cyclic Amplification (PMCA) is a technique which was developed to mimic the replication of TSE agents *in vitro* (Saborio et al., 2001). Each cycle is composed of two steps. In Step 1 small amounts of PrP^{Sc} are incubated with a large excess of PrP^C; this appears to induce the formation of co-aggregates of PrP^C and PrP^{Sc} and the subsequent conversion of at least some of the PrP^C to a protease-resistant PrP^{Sc}-like isoform within the co-aggregate. In Step 2 of the cycle sonication is used to break-up the co-aggregate of PrP^{Sc}, PrP^C and the newly-formed PrP^{Sc}-like isoform into a larger number of PrP^{Sc} aggregates, thus multiplying the number of growth sites for subsequent conversion. With each successive cycle, there is an increase in the number of these “seeds” and thus the conversion process is accelerated (Saa et al., 2006). The cyclic nature of the system permits the use of as many cycles as it is required to reach the amplification state needed for the detection of PrP^{Sc} in a given sample. PCMA has a high sensitivity for detecting PrP^{Sc} and, in some cases, it has been possible to amplify amounts of PrP^{Sc} in samples equivalent to one biological infectious unit (Saa et al., 2006) to a level that can be detected by conventional Western Blot or ELISA techniques. The *in vitro* generated PrP^{Sc} molecules seem to possess the most important key biological features of TSE agents:

- They are infectious *in vivo* (Castilla et al., 2005). These studies were done in hamsters (Castilla et al., 2005), mice (Castilla et al., 2008a), voles and other wild rodents (Fernández-Borges et al., 2009), deer (Green et al., 2008c), sheep and humans (Castilla et al., 2008b; Soto et al., 2005). Recombinant bacterial prion protein including RNA and lipids has recently been converted to TSE agents capable of infecting mice (Wang et al., 2010) or hamsters using PMCA without any mammalian or synthetic cofactors (Kim et al., 2010).
- The strain specificity of the PrP^{Sc} used initially to seed these reactions is conserved on *in vitro* replication (Castilla et al., 2008b). These studies were done using several mouse strains (301C, 79A, 139A, Me7, RML), human strains (Castilla et al., 2008b) and deer strains (Green et al., 2008c).
- PMCA and transmission barriers. The relative ease of conversion of heterologous PrP^C (PrP^C of a different species or primary amino-acid sequence from the PrP^{Sc}) by PrP^{Sc} in PMCA appears to correlate with the ability and efficiency of the equivalent (to the PrP^{Sc}) TSE agents to infect (and produce disease) in the heterologous species. The performance of PMCA related to this type of transmission barrier was studied for the first time when mouse TSE agents were subjected to serial heterologous PMCA rounds in order to be converted in hamster TSE agents (Castilla et al., 2008a). As it is happening *in vivo*, the new *in vitro* generated hamster TSE agent showed similar but not identical characteristics to other hamster TSE agents (263K, HY and DY). To date, the correlation between the *in vitro* and *in vivo* properties of PrP^{Sc} is only qualitative and has not yet been useful in predicting the likelihood of cross-species transmission of animal TSEs to humans.
- New TSE agents. PMCA has proved to be a technique able to generate TSE agents with apparently novel properties (Castilla et al., 2008a). The most striking example of this is the *in vitro* generation of TSE agents capable of infecting rabbits using PMCA (Fernández-Borges et al., 2009). Several attempts had failed to produce different TSE agents in rabbits and there are no documented cases of transmission of natural isolates of TSE agents to these lagomorphs (Barlow and Rennie, 1976; Gibbs and Gajdusek, 1973).
- Evaluating the effect of intermediate hosts or bridging species using PMCA. There are several examples of TSE agents in species A transmitting to species B only after intermediate passage in species C. For example, CWD in deer transmits to Syrian hamsters only after passage in ferrets (Bartz et al., 1998). PMCA mimics these phenomena and can be used to infer the possible consequences of exposure of humans to TSE agents via an intermediate host (Jones et al., 2009).

Technical modification of the PMCA technique by replacing sonication and serial reactions by periodic shaking has been shown to amplify low levels of PrP^{Sc} to levels that allow its detection by conventional assays: this Quaking-Induced Conversion (QUIC) assay may solve some of the practical drawbacks of applying PMCA as a routine diagnostic assay for TSE agents in tissues and body fluids (Atarashi et al., 2007).

Overall Conclusions on conversion assays:

- Cell-free conversion and PMCA (or modifications such as res-PMCA and QUIC) allow the *in vitro* propagation of a panel of TSE agents. So far, most TSE agents propagated through PMCA retain the biological specificities of the original agents.
- The ability to create TSE agents by *in vitro* conversion assays with a novel or unprecedented host range (such as those that can infect rabbits) indicate that there is probably no absolute molecular barrier to transmission of TSE agents between mammalian species.
- The qualitative correlation between *in vivo* data and *in vitro* results suggests that *in vitro* conversion assays may be developed as a tool for quantifying the transmission barriers between diverse species and for different TSE agents. However, there is at the moment no means by which to calibrate and transpose the ease of heterologous conversion *in vitro* into the likelihood of transmission between species *in vivo*.

3.3.3. Cell culture

Cell culture models are useful tools to study TSE cellular biology. They have recently been used as alternative tools to mouse bioassays to estimate infectivity from murine (Klohn et al., 2003), sheep scrapie (Neale et al., 2010) or CWD (Bian et al., 2010) infectious sources. In this paradigm, the abilities of TSE agents to develop in different mice cell lines have been proposed as a tool to differentiate between murine-adapted TSE strains (Mahal et al., 2008). The mechanisms underlying the selectivity of cellular models towards TSE agents remain unknown.

Although primary cultures of neurons from human PrP transgenic mice appear to propagate sCJD agent (Cronier et al., 2007), there is currently no cellular model expressing human PrP that would allow a long-term, efficient propagation of human TSE agents or of third species isolates (Crozet et al., 2008; Vilette, 2008). Consequently it is not possible to foresee in the next future the use of cellular models as an approach for evaluating the capacity of TSE agents to cross the human transmission barrier.

4. Assessment according to agent

4.1. Classical BSE

4.1.1. Epidemiology

Currently the most relevant world-wide epidemiological pattern is a general and constant decline in the frequency of BSE in the field where sufficient surveillance data are available.

The BSE epidemic spans over 25 years with an overall number of cases as to 31 December 2009 of 190,552 (184,600 of them occurring in the UK). These data were obtained from the OIE website, from national official websites and from the reports on the monitoring and testing of ruminants for the presence of TSEs in the EU¹¹: it is likely that the figures do include Atypical BSE cases, but due to their low numbers in most cases they hardly affect the statistics regarding Classical BSE.

After the first year of EU-wide enforcement of the active surveillance (2001), the number of cases increased dramatically: in 1999 the cases of BSE identified outside the UK had been 340, in 2001 they were 1,015, with eight countries, including Japan, reporting the disease for the first time.

Between 2001 and 2009, most BSE cases (98.1%, i.e. 7,781 out of 7,934) came from the EU, about 50% of them from the UK (3,755). In the rest of the world, over the 2001-2009 period, the number was really small (98 in Switzerland, 36 in Japan, 16 in Canada, 2 in US and 1 in Israel). However, the large European figures, compared with those coming from the rest of the world, are also due to the huge surveillance effort that, between 2001 and 2008, resulted in having over 80.9 million cows tested.

Unfortunately the passive nature of surveillance, based on the mandatory reporting of clinical cases, in the decade before 2001 makes the available data useless for reliable interpretations.

The average annual crude incidence by country (accounting for both period of observation since the enforcing of the active surveillance, for most countries 2001-2009, and the size of the adult cattle population) may be used to show the magnitude of the national BSE epidemics (See Figure 1). Although the crude rates may be confounded by different population structures (e.g. in terms of age distribution), by differences in the sampling scheme (exhaustive in most EU Member States since 2001 vs. sample-based in the rest of the world where active surveillance was applied) or by the effectiveness of the surveillance applied, they may provide a summary description of the worldwide situation which is consistent with that provided by Ducrot et al. (2008). In order to allow comparisons, in Figure 1 the calculation has been restricted to European countries where an exhaustive sampling scheme has been applied: Portugal, Ireland and Spain experienced the largest epidemics although with lower incidence than in the UK. In most of the countries where a sample-based scheme was applied, the incidence appears to be lower (average annual cases per million cattle over 24 months respectively: the UK 78.72, Switzerland 11.43, Japan 2.01, Israel 0.78, Canada 0.41, Sweden 0.37, US 0.01); however, the different sampling approach likely led to an important underestimation of the real incidence.

Looking at the trends of the number of BSE cases per year may help in identifying the country-specific trend of the human exposure to BSE. In the period between 2001 and 2009, the overall fading out of the epidemic is consistent in Western Europe (i.e. in Switzerland, in the UK, in the remaining countries of the EU15) whereas in the rest of the world (Northern America, Japan and the EU new

¹¹ Source: European Commission, available at http://ec.europa.eu/food/food/biosafety/tse_bse/monitoring_annual_reports_en.htm

Member States), the epidemic reached a peak in 2005-2006 before starting the recent decline (See Figure 2). The size of epidemics and peak years by country in Europe is shown in Figure 3: there are three waves involving, respectively, in the first the UK, Portugal and Switzerland before 2001, in the second most of the EU Member States between 2001 and 2003, and in the third Poland, Czech Republic and Slovakia whose epidemics peaked around 2005.

There are caveats to consider for the country-specific characteristics of the surveillance, which may be applied very differently and lead to biased estimates of the national level of BSE. These characteristics may for instance be in terms of: mainly active or passive, on samples or on the entire at risk populations, targeted/risk based (ratio fallen stock to regular slaughtered animals). Moreover differences may exist in the diagnostic techniques applied and therefore in the overall sensitivity of the surveillance system (e.g. in the ability of detecting atypical cases). As a result, the surveillance applied may differ in its overall intensity and therefore in its ability of detect/quantify the real frequency of the disease; the OIE point values system may help in an overall interpretation of BSE national data, however risk assessment results may deserve further consideration. Moreover, confounding factors need to be considered as well. Currently only crude (i.e. non standardized) statistics are published and available for comparison: the structure of ruminant populations submitted to monitoring are not taken into account, e.g. ruminants populations that differ in the distribution of age or breed are not directly comparable.

Finally, estimating the real prevalence of infection within the population is confounded by the differential survival of cattle. Back-calculation studies (Ferguson and Donnelly, 2003; Supervie and Costagliola, 2004) have shown that many preclinically or subclinically infected animals may not be detected by passive and/or active surveillance because they die or are culled before signs of disease or infection can be detected by diagnostic methodologies. For instance, it has been estimated that in the UK, compared to the 180,000 cases identified up to 2002 by surveillance, the number of infected cattle was about four million (Ferguson and Donnelly, 2003). Similarly, between July 1980 and June 1997 in France, Supervie and Costagliola (2004) estimated that about 300,000 cattle were infected by the BSE agent although only a small fraction of these animals survived to develop disease or were culled at a time when markers of infection could be detected *post mortem*.

4.1.2. Pathogenesis

Bovine spongiform encephalopathy (BSE) was recognized as a cattle TSE disease during the 1980s (Wilesmith et al., 1988) in the UK. Early studies indicated that the BSE agent can only be found in the brain, spinal cord and retinal tissue of BSE-diseased cattle. Infectivity assessment in several tissues from orally inoculated cattle, using bioassays based on RIII mice (Wells et al., 1994; Wells et al., 1998), revealed BSE infectivity in the CNS, all brain regions, the spinal cord, the optic nerve, the retina (neuronal cells), cervical, thoracic and trigeminal ganglia, and the facial and sciatic nerves, as well as in distal ileum. The skeletal muscles, spleen and other lymphatic tissues were shown to be free of detectable infectivity. More recently, Wells et al. (2005) showed infectivity in tonsil tissue from cattle killed 10 months after oral BSE challenge by intracerebral inoculation in cattle.

Experiments in transgenic mice overexpressing bovine PrP confirmed the essential restriction of infectivity to the nervous system, distal ileum and tonsils in terminally BSE-diseased cattle (Buschmann and Groschup, 2005) as well as in asymptomatic cattle at different times (20–33 months) after oral challenge (Espinosa et al., 2007b). All together these results are consistent with the idea that BSE infectivity, after oral uptake, propagates only poorly in some intestinal lymphatic tissues (mainly Peyer's patches) and from there spreads to the CNS, probably by intraneural spread via the peripheral nervous system. Titres are relatively low or undetectable outside the CNS.

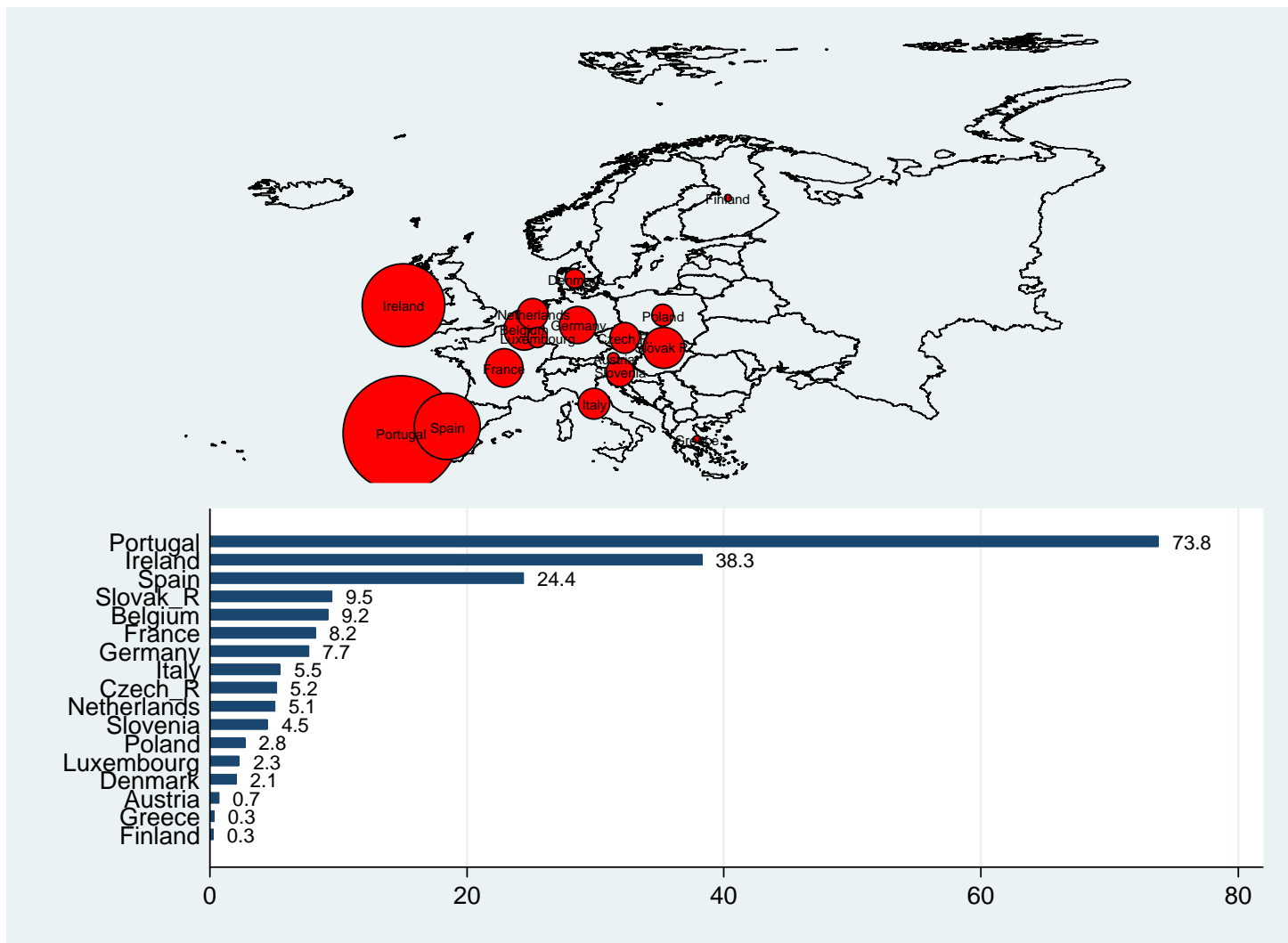


Figure 1: Crude average annual incidence by country (in terms of cases per million cattle over 24 months per year, calculated over the 2001-2009 period) in Europe. The Figure includes only countries where an exhaustive sampling scheme has been applied (i.e. data for UK and Switzerland are not reported).

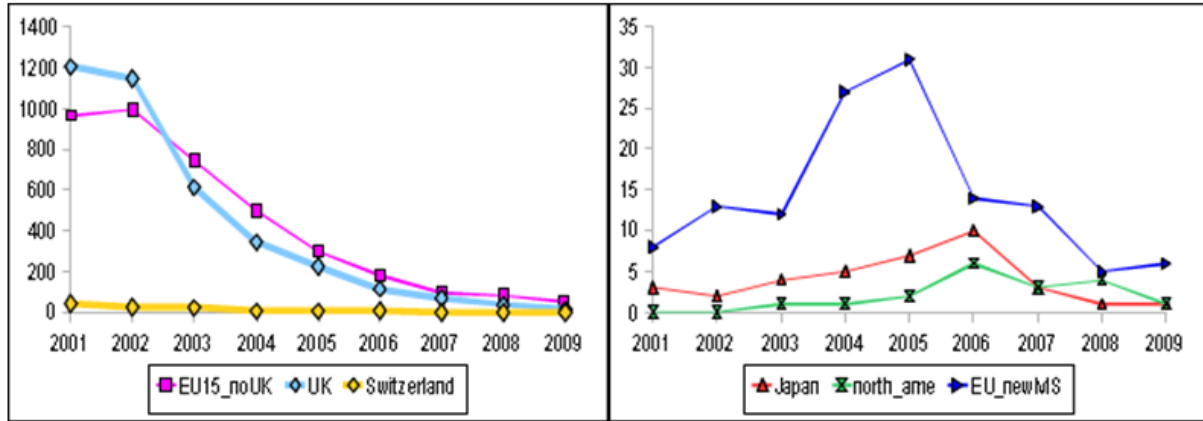


Figure 2: Trends of the number of cases per year between 2001 and 2009 in the UK, Switzerland, EU15 without the UK (Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden), EU new Member States (Czech Republic, Poland, Slovenia, Slovakia, where cases of BSE were reported), and Northern America (US and Canada).

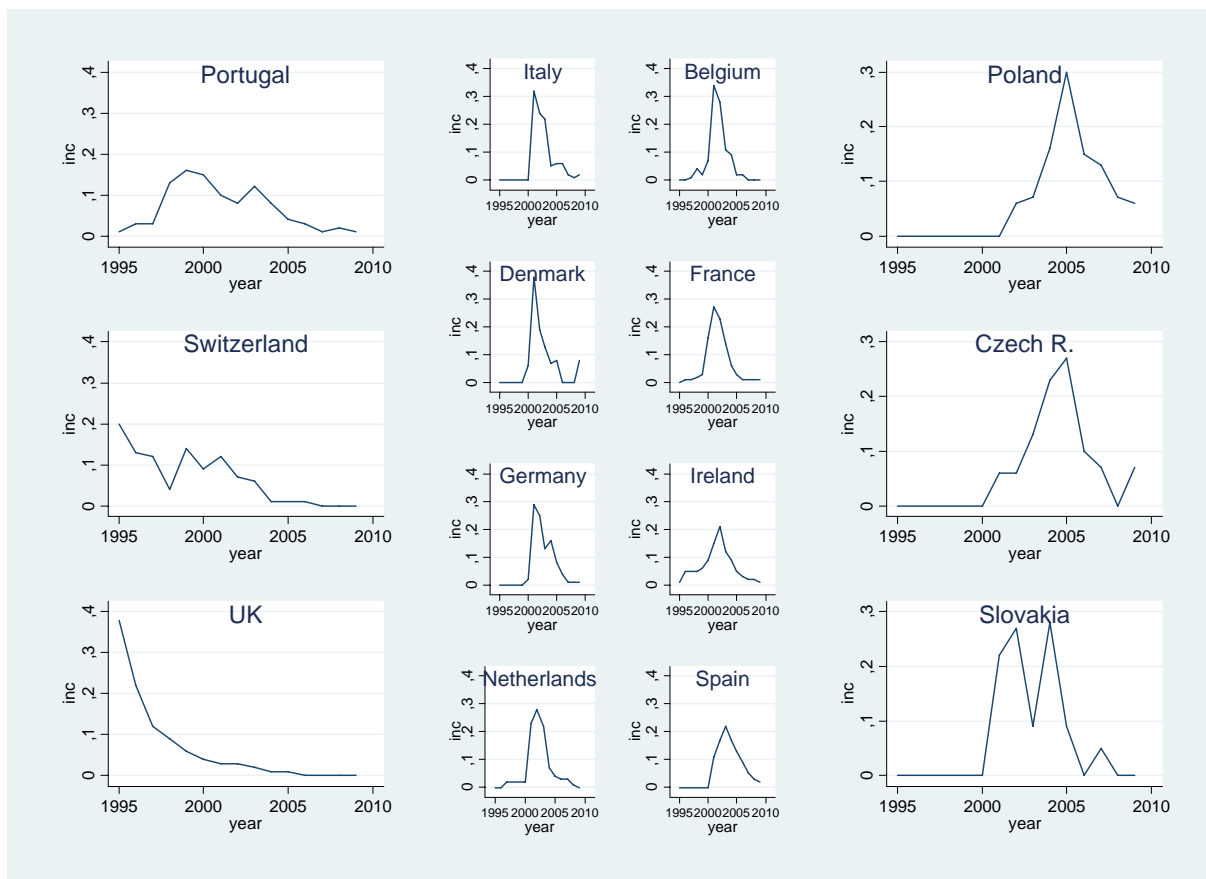


Figure 3: Country- and year-specific crude incidence in Europe (countries with less than a total of 15 cases not shown). The incidence rates have been normalized, i.e. overall incidence for the period 1995-2009 = 100%.

These findings are in contrast to the spreading of the scrapie agent in infected sheep, mice and hamsters in tissues such as spleen, other lymphatic tissues, muscles etc., even during the preclinical stage (Bosque et al., 2002; Heggebo et al., 2003; Thomzig et al., 2003; Thomzig et al., 2004). However, the BSE agent can be found in the lymphoreticular system and other peripheral tissues when transmitted to sheep or primates (Andreoletti et al., 2006; Bons et al., 1999; Herzog et al., 2004; Jeffrey et al., 2001).

4.1.3. Transmission *in vivo* and *in vitro*

BSE is considered a highly promiscuous TSE strain, transmitting efficiently experimentally by intracerebral route to several species including mice, sheep, goats, pigs, cats, mink and primates.

Experimental transmission to a common, “reporter” species has highlighted the remarkable ability of the BSE agent to retain its biological properties after intermediate passage in a range of different hosts with distinct PrP^C sequences. In initial studies, transmission of various sources of infected cattle to a panel of inbred mice expressing the a or b mouse PrP allele suggested that cattle have been infected by a single strain since incubation periods and distribution of spongiosis in the brain were uniform in each genotype, unlike that seen with scrapie or CJD isolates (Bruce et al., 1994; Bruce et al., 1997; Green et al., 2005). The two agents propagated on a and b genotypes were termed 301C and 301V, respectively. Strikingly, 301C and 301V were invariably obtained irrespectively of the species infected by BSE agent, either accidentally (cats, exotic ruminants, humans) or experimentally (sheep, goats, pigs, macaques) (Bruce et al., 1994; Bruce et al., 1997; Lasmezas et al., 2001). It should be kept in mind, however, that the BSE agent underwent a single passage through the intermediate host. It remains unclear whether a more thorough adaptation to these species would lead uniformly to a conservation of the strain phenotype, as continued passage in a new host can alter strain characteristics e.g. 301V and 301C (Bruce et al., 1994). Surprisingly enough, a divergent evolution of the BSE agent has been reported following transmission to various lines of inbred mice, all carrying the *PRNP*-a allele (Asante et al., 2002). Careful phenotype comparison confirmed the presence of two distinct mouse strains, one resembling to the Chandler strain, the other to mouse BSE (Asante et al., 2002; Lloyd et al., 2004). This is an important observation as it suggests that other loci than PrP might influence not only the susceptibility (Lloyd et al., 2004; Lloyd et al., 2001) but also the strain evolution. Puzzlingly, however, these results have not been reproduced in another study using the same panel of mice and different BSE isolates (Capobianco et al., 2007).

More recently BSE agent stability after passage through sheep species has been further investigated. The transmission features were compared with cattle BSE in mice transgenic for bovine PrP (Espinosa et al., 2007a). While the molecular and pathological phenotypes were indistinguishable, sheep-passaged BSE induced a significantly shorter incubation period on the first but also subsequent passages in bovine transgenic mice, thus excluding different infectivity levels in cattle and sheep brains as a possible explanation. An increased pathogenicity of sheep-passaged BSE was also observed in mice transgenic for porcine PrP (Espinosa et al., 2009), as well as in conventional RIII mice when compared with retrospective cattle BSE experiments (Gonzalez et al., 2007). These data raise the possibility that BSE agents may gain virulence by passage in another species.

Experimental transmission of cattle BSE isolates to transgenic mice expressing methionine at human PrP codon 129 revealed a very low transmission efficiency with low attack rate and long incubation times, suggesting a strong transmission barrier for cattle BSE. This low BSE transmission efficiency to human PrP transgenic mice is occasionally accompanied by a strain shift allowing the appearance of an alternative, sporadic CJD-like phenotype in a proportion of mice (Asante et al., 2002; Bishop et al., 2006). Although the exact characteristics and further evolution of the vCJD epidemic still entail uncertainties owing to prolonged incubation times, this modelled high transmission barrier of humans to cattle BSE might be an explanation for the currently low vCJD incidence, considering the high exposure to BSE during the “mad cow” crisis.

Sheep and goats are experimentally susceptible to BSE (Foster et al., 1993; Houston and Gravenor, 2003). During the BSE epidemic, sheep and goats have been exposed to BSE-contaminated meat and bone meal (MBM), so BSE transmission to these species may have occurred. A recent study (Padilla et al., In press) evaluated the human susceptibility to small ruminants-passaged BSE agents by inoculating two different transgenic mouse lines expressing the methionine (Met) allele of human PrP at codon 129 (tg650 in INRA-France and tg340 in INIA-Spain) with several sheep and goat BSE isolates and compared their transmission characteristics with those of cattle BSE. In this study, the transmission efficiency of cattle BSE isolates in both human-PrP transgenic mouse models was low (very low attack rates and long incubation times). Remarkably, a different picture emerged when the sheep and goat BSE isolates were inoculated into human PrP transgenic mouse models. Attack rates approaching 100% were observed from the primary passage onwards and mean incubation times were more consistent with those measured after transmission of vCJD.

These results clearly indicate that Met129 homozygous individuals might be susceptible to a sheep or goat BSE agent at a higher degree than to cattle BSE, and that these agents might transmit with molecular and neuropathological properties indistinguishable from those of vCJD. These data suggest that the possibility of small ruminant BSE agents as causal agent for vCJD could not be ruled out. Against the small ruminant BSE as vCJD causal agent hypothesis is the fact that only two natural BSE cases have been reported in goats and none in sheep (Eloit et al., 2005; Jeffrey et al., 2006). Epidemiological studies, mathematical models and strain typing studies in mice described that the presence of BSE in the sheep population cannot be excluded, predicting proportions of BSE among sheep TSEs between 0,06 and 5% (Gravenor et al., 2003; Stack et al., 2006). A most recent EFSA Opinion on BSE/TSE infectivity in small ruminant tissues (EFSA Panel on Biological Hazards (BIOHAZ), 2010a) concluded that with 95% confidence the the number of BSE infected animals that could enter yearly into the food chain in the EU is ranging between 0 and 240 for sheep and between 0 and 381 for goats. Although these numbers are relatively low, the susceptibility of humans to a sheep BSE agent, suggested being higher than to bovine BSE has important considerations for public health. Since in BSE infected sheep PrP^{Sc} has been detected in peripheral organs such as tonsil, retropharyngeal lymph node, ileo-caecal and mesenteric lymph nodes, sheep might be a more dangerous source of BSE infectivity for man as compared to cattle (Bellworthy et al., 2005; Foster et al., 2001; Jeffrey et al., 2001). In the same way, a mathematical model analysis, estimating the human health risk from possible BSE infection of the British sheep flock, warns about public health risks higher than those from cattle BSE (Ferguson et al., 2002). This fact is even more worrying since transmission studies suggest that apparently Met129 human PrP^C prefers a BSE agent with ovine rather than a bovine sequence. Finally, it is evident that, although few natural cases have been described and so far we cannot draw any definitive conclusion about the origin of vCJD, the risk of a potential goat and/or sheep BSE agent should not be underestimated.

Several transmission experiments in primate models were performed to assess the risk of BSE for human health. Lemurs, marmosets, macaques and squirrel monkeys developed spongiform encephalopathies after intracerebral inoculation of brains from BSE-infected cattle (Baker et al., 1993; Bons et al., 1999; Lasmezas et al., 1996; Williams et al., 2007). Secondary transmission to the same host, i.e. conventional mice, of both macaque BSE and human vCJD induced similar lesional profiles, bringing an additional evidence for the similitude between BSE and vCJD agents (Lasmezas et al., 2001).

Subsequently, lemur and macaque models demonstrated the transmissibility of BSE through the oral route (Bons et al., 1999; Lasmezas et al., 2005). In macaque, 5 grams were sufficient to transmit the disease to one of two inoculated animals (Lasmezas et al., 2005). Furthermore, risk of secondary transmission through transfusion was assessed in the same primate models: infectivity of blood components was demonstrated through intracerebral inoculation in lemurs (Bons et al., 2002), the intravenous route was demonstrated as an efficient way of transmission in macaques (Herzog et al., 2004), and finally transmission was achieved through transfusion in this latter model (Comoy et al., 2008a).

In conclusion, those different transmission experiments to primates supplied useful evidence for risk assessment of BSE for human health on one hand, but also stressed the relevance of those primate models for modelling the human situation.

Conclusions:

- Currently the most relevant world-wide epidemiological pattern is a general and constant decline in the frequency of Classical BSE in the field where sufficient surveillance data are available.
- There is converging evidence that the Classical BSE epidemic was caused by a single major agent which had the ability to cross different species barriers under natural exposure conditions.
- Experimental transmission to mice and primate models has shown that the Classical BSE and vCJD agents share specific features.
- Transmission of cattle Classical BSE to human PrP transgenic mice together with the relatively limited size of the vCJD outbreak suggests existence of a substantial transmission barrier.
- Transmission studies in human PrP transgenic mice (Met129) suggest that sheep or goat Classical BSE agent might have a higher potential than cattle Classical BSE to propagate in humans.

4.2. Atypical BSE

4.2.1. Epidemiology

The large-scale testing of livestock nervous tissues for the presence of PrP^{Sc} has led to the recognition of two molecular signatures distinct from BSE in cattle. These were termed H-BSE and L-BSE (or BASE), respectively. Their PrP^{Sc} molecular signature differed from Classical BSE in term of protease-resistant fragments size and glycopattern (Biacabe et al., 2004; Buschmann et al., 2006; Casalone et al., 2004). The experimental transmission of these cases to different lines of bovine PrP transgenic mice unambiguously demonstrated their infectious nature and confirmed their unique but distinctive strain phenotype as compared to BSE (Beringue et al., 2007; Beringue et al., 2006; Buschmann et al., 2006; Capobianco et al., 2007).

So far, L-BSE has been identified in France (Biacabe et al., 2008), Italy (Casalone et al., 2004), Germany (Buschmann et al., 2006) and Poland (Polak et al., 2008); H-BSE in France (Biacabe et al., 2008), Great Britain (Terry et al., 2007), Germany (Buschmann et al., 2006), the Netherlands (Jacobs et al., 2007), the USA (Richt et al., 2007), Sweden (Gavier-Widen et al., 2008) and Switzerland (in a zebu (Seuberlich et al., 2006)).

These uncommon cases have been essentially detected in aged asymptomatic cattle during systematic testing at slaughterhouse. In France a retrospective study of all TSE-positive cattle identified through the compulsory EU surveillance programme between 2001-2007 was performed (Biacabe et al., 2008).

This study indicated that:

- All H-BSE and L-BSE cases detected by rapid tests were observed in animals over eight years old in either the fallen stock surveillance stream or the abattoir (healthy slaughter).
- No H-BSE and L-BSE were observed in the passive epidemio-surveillance network although, during retrospective interviews, the farmers and veterinarians for six of these animals reported clinical signs consistent with TSE in three fallen stock.

- Frequency of H-BSE and L-BSE is respectively 0.35 and 0.41 cases per million adult cattle tested but increases to 1.9 and 1.7 cases per million in over eight years old animals tested.

The number of Atypical BSE cases detected in countries that have already identified them seems to be comparable from year to year. No comprehensive study on the prevalence of Atypical BSE cases has been done in other EU Member States and the performances of the currently available rapid test applied for initial TSE screening of the cattle population towards Atypical BSE is still unknown.

The origin of these Atypical BSE cases in cattle is currently unknown, as is the performance of the current active surveillance system for detecting H-BSE and L-BSE affected animals, resulting in uncertainty about the real prevalence of these conditions.

All Atypical BSE cases identified in the EU were born before the extended or real feed ban that came into law in January 2001 (Ducrot et al., 2008). Hence, as with Classical BSE, exposure of these animals to feed contaminated with low titres of TSE agents cannot be excluded, although other origins for these TSE forms cannot be discarded. In particular, the unusually old age of all H-BSE and L-BSE identified cases and their apparent low prevalence in the population could suggest that these Atypical BSE forms are arising spontaneously.

4.2.2. Pathogenesis

To date there is no comprehensive information about the pathogenesis and cause (spontaneous disorder or acquired infectious agent) of Atypical BSE in cattle.

There are however some data related to the peripheral distribution of L-BSE agents in cattle experimentally challenged by the intracerebral route. In one Japanese study, infectivity and PrP^{res} were detected in many peripheral nerves tested from mid-incubation onwards. The protein was not detected in lymphoid tissue (Iwamaru et al., 2010). In one Italian study, PrP^{res} was not detected in peripheral nerves (Lombardi et al., 2008), but presence of infectivity in skeletal muscle, presumably linked to nervous structures, of natural cases has been described (Suardi et al., 2009).

4.2.3. Transmission *in vivo* and *in vitro*

Both L-BSE and H-BSE agents are able to propagate in experimentally challenged foreign species such as mouse, sheep, vole, primates and hamster and in transgenic mice expressing heterologous, i.e. non-bovine PrP, sequences.

Noticeably, the transmission barrier observed for the L-BSE agent was lower than that for epizootic BSE. In wild-type mice and in transgenic mice expressing the VRQ allele of ovine PrP, the L-BSE agent acquired a phenotype undistinguishable from the BSE agent (Beringue et al., 2007; Capobianco et al., 2007).

More recently transmission of H-BSE isolates originating from France and Poland to bovine-PrP transgenic mice has been reported. While in the majority of the cases the propagated TSE was different from Classical BSE, Classical BSE has emerged in a proportion of the inoculated mice inoculated with two distinct isolates (one from France and one from Poland) (Espinosa et al., 2010).

Together these data indicate that there may be an aetiological relationship between Atypical and Classical BSE.

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., 2008b). In the frame of a primary passage through inoculation of a same amount of infected brain, incubation periods were shorter (23-25 months) than for BSE (38-40

months), suggesting that L-BSE may be more virulent than Classical BSE for infecting primates. L-BSE was also transmissible to microcebes, with shorter incubations than Classical BSE (Baron et al., 2008). Moreover, recent experiments demonstrated the transmissibility of L-BSE to macaque by the oral route (Comoy, 2010) with 5 grams of infected brain, this amount being similar to the one used for oral transmission of Classical BSE in the macaque model.

Histology and biochemistry studies showed similarities between L-BSE-inoculated macaques and MM2 sporadic Creutzfeldt-Jakob disease patients: infected primates and those rare patients exhibited similar lesional profiles, and their respective PrP^{res} showed the same sensitivity of their N-terminal parts to proteolysis. Moreover, a macaque inoculated with brain of a MM2 sCJD patient showed similar lesional profile as L-BSE infected macaques (Comoy et al., 2009).

In contrast, no clinical sign has been observed 72 months after intracerebral inoculation of brain from H-BSE, and recipient cynomolgus macaques remained healthy, suggesting a lower, if any, virulence of this agent for primate (Comoy, 2010).

The intracerebral inoculation of L-BSE field isolates produced TSE disease in two lines of mice overexpressing human PrP (Met129), exhibiting a molecular phenotype distinct from Classical BSE (Beringue et al., 2008a; Kong et al., 2008). In one of them, the L-BSE agent appeared to propagate with no obvious transmission barrier: a 100% attack rate was observed on first passage, the incubation time was not reduced on subsequent passaging (Beringue et al., 2008a), and the L-type PrP^{Sc} biochemical signature was essentially conserved (Beringue et al., 2008a; Kong et al., 2008). The latter appeared undistinguishable from that seen after experimental inoculation of MM2 sCJD in these mice (Beringue et al., 2007). These transmission features markedly differed from the low transmission efficiency of cattle BSE isolates to this (Beringue et al., 2008a; Beringue et al., 2008b) and other (Asante et al., 2002) human PrP transgenic mouse lines.

H-type isolates failed to infect one line of “humanized” mice (Beringue et al., 2008a). These mice overexpress human PrP and were inoculated intracranially with a low dilution inoculum, supporting the view that the transmission barrier of H-type BSE from cattle to humans might be quite robust.

The permissiveness of “humanised” transgenic mice expressing the valine allele at codon 129 (or hemizygous) to Atypical BSEs is currently unknown.

Conclusions:

- The true incidence and geographical distribution of atypical forms of BSE has not been established.
- Both L-BSE and H-BSE have shown BSE-like characteristics on transmission studies in some lines of mice. The precise relationship between Classical BSE, H-BSE and L-BSE is not yet clear. However these experiments have shown that the potential for interspecies transmission of Atypical BSE is high.
- Several elements indicate that the L-BSE agent has the potential to be a zoonotic agent. Primates are highly permissive to L-BSE agents, even by the oral route, and these can also propagate without any apparent transmission barrier in transgenic mice overexpressing human PrP.
- 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 transmissible to mice overexpressing the Met allele of human PrP, nor to primates.

4.3. Classical scrapie

4.3.1. Epidemiology

Scrapie in small ruminants is a disease described for several centuries. It was reported for the first time in UK in 1732 and affects both sheep and goats (Detwiler, 1992). The disease has been reported in a large number of countries in Europe, Asia and America. According to the OIE, Australia and New Zealand are Classical scrapie free countries.

During decades the identification of Classical scrapie has relied on clinical suspicion (passive surveillance). In 2002, in the EU, an active epidemio-surveillance system has been implemented: this system is based on the systematic testing of a proportion of the slaughtered or found-dead animals for PrP^{Sc} presence in the posterior brainstem.

The data collected through this surveillance system have clearly demonstrated that earlier perceptions on the prevalence of TSEs in small ruminants and their geographical spreading (on the basis of passive surveillance) were inaccurate.

Active surveillance has provided a better picture of the apparent disease prevalence in EU Member States. Both European sheep and goats, although the latter species to a lower degree, have been involved by the disease. Limiting the description to the ovine population, in the EU, since the implementation of the sample-based testing, about 3.5 million sheep were tested; up to 2008 the active surveillance was able to identify over 4.7 thousand cases. Most countries were involved (21 out of 27) with remarkable geographical differences and a large outbreak in Cyprus. Excluding the latter, over the 2002-2008 period the EU-wide apparent crude prevalence was equal to about 11 cases per 10,000 tested animals (7 and 16 among healthy slaughtered animals and fallen stock respectively). These data are a likely underestimation of the true prevalence as a proportion of outbreaks is missed. Finally the within-flock prevalence was on average 20 times higher than the apparent prevalence in the general population identified by active surveillance.

A most recent EFSA Opinion on BSE/TSE infectivity in small ruminant tissues (EFSA Panel on Biological Hazards (BIOHAZ), 2010a) concluded that, on the basis of data collected between 2007 and 2009, the total number of Classical scrapie infected animals that could enter yearly into the food chain in the EU27 as a whole was estimated to approximately range between 16,000 and 67,000 (most probable estimate 29,000) for sheep and between 10,000 and 34,000 (most probable estimate 13,000) for goats.

Despite the substantial progress it represents, active surveillance has been applied to a limited proportion of the animal population and over a relatively short (by comparison to a TSE incubation period in humans) period of time. Moreover, TSE cases occurring in small ruminants that could be differentiated from BSE, including Atypical scrapie, are reported under the category of “Classical scrapie”. This term thus encompasses a variety of TSE agents which harbour distinct biological properties (such as the capacity to be transmitted to hosts of different species).

This situation can partly be explained by the inability of the currently available TSE tests to differentiate between the different agents (which diversity remains unknown) causing Classical scrapie. It would however be inappropriate to consider in epidemiological analysis that all the agents causing Classical scrapie display the same abilities to cross transmission barriers.

4.3.2. Pathogenesis

Classical scrapie is an infectious disease of small ruminants for which susceptibility is influenced by polymorphisms on the gene (PRP) encoding for PrP protein (EFSA, 2006). In sheep, the major polymorphisms associated with susceptibility or resistance are codons 136 (A or V), 154 (R or H) and

171 (R, Q or H) (Cloucard et al., 1995; Hunter et al., 1996). VRQ/VRQ, ARQ/VRQ and ARQ/ARQ genotype animals are considered as the most susceptible to Classical scrapie, whereas homozygous or heterozygous AHQ and heterozygous ARR animals only show a marginal susceptibility. AHQ allele carriers as well as ARQ/ARQ sheep were described to be the most susceptible genotype to experimental BSE, while VRQ/VRQ were reported to be of lower susceptibility. ARR/ARR sheep are considered to be strongly (but not absolutely) resistant to Classical Scrapie (Hunter et al., 1996; Hunter et al., 1997). In goats other PrP polymorphisms (e.g. I/M142- H/R154- R/Q211- D/S 146 and Q/K222) could also impact on individual susceptibility to these TSE agents (Barillet et al., 2009; EFSA, 2009b; EFSA Panel on Biological Hazards (BIOHAZ), 2009; Gonzalez et al., 2009; Papasavva-Stylianou et al., 2007; Vaccari et al., 2006).

It is widely accepted that natural contamination with Classical scrapie in affected flocks mainly occurs around birth (Andreoletti et al., 2002; Race et al., 1998; Tuo et al., 2002). Contamination with Classical scrapie was reported in sheep that were introduced in an infected flock after they reached adulthood (Ryder et al., 2004). The efficacy of transmission appears to be lower in older animals than in younger ones. The origin of such contamination remains unclear and both inter-individual horizontal transmission and environmental sources could be at their origin. The role of the environment as a source of contamination is now unambiguously demonstrated (Dexter et al., 2009) by infection of naïve animals that were introduced to an infected environment without contact with animals, even after several weeks of the pasture being unpopulated.

Most of the available data related to Classical scrapie agent dissemination in small ruminants naturally infected were obtained in VRQ/VRQ sheep born and raised in two individual flocks (one in the Netherlands and one in France, Langlade flock).

According to these studies infection apparently occurs via the Gut Associated Lymphoid Tissues (GALT) before a rapid spread of the agent to draining mesenteric lymph node (Andreoletti et al., 2000; Andreoletti et al., 2002; Heggebo et al., 2000; van Keulen et al., 2002) and later to all lymph nodes, including those that remain on prepared carcasses (Andreoletti et al., 2000; Andreoletti et al., 2002; van Keulen et al., 2002). The amount of PrP^{Sc} in lymphoid formations increases with age before reaching a plateau level.

The TSE agent disseminates to the CNS (brain and spinal cord) apparently via the Enteric Nervous System and its nerves fibers (Andreoletti et al., 2000; Jeffrey et al., 2001; van Keulen et al., 2002), which is considered to accumulate TSE agents until around half of the incubation period. From there the agent could redistribute (centrifugally) to the peripheral nervous system and skeletal muscle (Andreoletti et al., 2004). Additionally, infectivity was also reported to be present in blood (Hunter et al., 2002), and in blood and in milk and colostrum (from the first lactation) from animals during incubation (Konold et al., 2008; Lacroux et al., 2008). In blood, the infectious agent can be detected as early as at 3 months of age and persists throughout the incubation period (Andreoletti et al., 2007).

This dissemination scheme is consistent with most of the data reported with regard to natural Classical scrapie cases in small ruminants. However VRQ/VRQ sheep are considered to be the most sensitive sheep PrP genotype to most of TSE agents responsible for Classical scrapie. In animals bearing different PrP genotypes and in goats the kinetics of the agent distribution in the organism of the affected animals can vary substantially. Moreover, under natural exposure conditions in heterozygote ARR sheep, the PrP^{Sc} distribution seems to be mostly confined to the CNS even if some minimal PrP^{Sc} deposits could be observed in the lymphoreticular system. Additionally in several Classical scrapie cases of ARQ/VRQ and ARQ/ARQ sheep (Jeffrey et al., 2002; Ligios et al., 2006) and goats (Gonzalez et al., 2009), PrP^{Sc} accumulation in CNS was reported in the absence of detectable PrP^{Sc} in the lymphoid tissues. Together these data indicate that if the Classical scrapie pathogenesis in sheep would have to be coherently considered at a global scale, our knowledge relies on the study of a limited number of flocks.

Therefore, considering the potential diversity of TSE agents that are responsible for Classical scrapie and the importance of the interaction between the host genotype and the TSE agent strain properties, these data cannot be considered to apply to all Classical scrapie cases.

Finally, protection measures applied all along the food chain against small ruminants TSEs in the EU mainly rely at operational level on specified risk material (SRM) removal, i.e. exclusion from food chain of tissues that can contain a high infectious load. However, for practical reasons, the SRM measures do not imply discarding from the food chain of all the infectious tissues and animal products that could contain infectivity (EFSA, 2008c). Moreover, infectivity was recently identified in tissues like skeletal muscles or in products like milk from small ruminants incubating scrapie, tissues that were previously considered to be non infectious.

Together these elements indicate that despite the protective measures implemented in 2001, infectivity from Classical scrapie agents has continued to enter into the food chain.

Relative infectious titers in tissues from natural scrapie infected sheep and goats vary according to the age and genotype of the host. Detailed information is provided in a recent EFSA opinion (EFSA Panel on Biological Hazards (BIOHAZ), 2010a).

4.3.3. Transmission *in vivo* and *in vitro*

The demonstration that scrapie can be due to different TSE agents has been recognized since the end of the 50's, using experimental transmission in natural hosts (Pattison and Millson, 1961a). Transmission experiments to inbred mice lines using field scrapie isolates confirmed the existence a certain number of differentiable strains (Baron et al., 1999; Baron et al., 2000; Bruce and Fraser, 1991; Bruce et al., 1991).

The comparison between two TSE isolates from hosts belonging to different species is made by transmission of these agents to a third species host (usually laboratory rodents). This approach has been at the basis of the TSE strain typing approach developed in Scotland over the 1960 and 70's. It revealed to be extremely useful for demonstrating that the same TSE agent was at the origin of BSE in cattle and vCJD in humans (Bruce et al., 1997). In this approach the isolates are passaged (several passages) in conventional inbred mice lines harbouring different PrP haplotypes.

Because of the existence of a transmission barrier, a significant proportion of the scrapie field isolates cannot be propagated in conventional mice models (Beck et al., 2010a; Bruce et al., 1997). Moreover the effect of the passage over this transmission barrier can impact on the TSE strain properties. Together these elements represent major limitations for describing the Classical scrapie biodiversity using conventional mice models.

Similarly, most of the sCJD isolates failed to propagate in these mice models (Bruce et al., 1997; Hill et al., 1997), which limits the use of conventional mice bioassay for comparing Classical scrapie and human TSE isolates with this approach.

Nevertheless in C57Bl6 mice a natural scrapie isolate was reported to exhibit, after first passage, a phenotype similar to sporadic and iatrogenic CJD (Lasmezas et al., 2001). However, considering the potential effect of transmission barrier passage on the strain properties, this result remains difficult to interpret.

More recently, bank voles (*Myodes glareolus*) have been proposed as a model to compare human and animal TSE isolates. Indeed both sCJD isolates and animal TSE isolates seem to propagate efficiently in this model (Nonno et al., 2006). To date data results published in this model have not indicated similarities between the investigated CJD cases and the ruminant TSE isolates. However, the limited diversity of the tested animal TSE isolates does not allow at this stage to draw definitive conclusions.

The creation of transgenic mice models expressing ovine PrP variants (natural PrP promoter or over-expressing models) provided new opportunities to study the biodiversity of scrapie agents (Beringue et al., 2008b; Vilotte et al., 2001). The development of various transgenic mice expressing different PrP protein variants offers new possibilities to investigate the relative potential abilities of Classical scrapie isolates to cross the “human transmission barrier”. The pertinence of transgenic animal models for characterizing the full diversity of TSE agents is still under discussion.

Some projects aiming at characterizing the capacity of field Classical scrapie isolates to pass “transmission barriers” are currently ongoing using transgenic mice models. To date, results from such experiments remain extremely limited and insufficient to draw any definitive conclusion. It has been observed that some natural sheep scrapie isolates can propagate with limited or no apparent transmission barrier in bovine PrP transgenic mice (Scott et al., 2005). There are no published results on transmission of Classical scrapie agents to human PrP transgenic mice yet, although studies are ongoing in different laboratories.

A very limited number of Classical scrapie isolates has been inoculated into primates so far. The infectious material was either originating directly from small ruminants or from field isolates that were propagated in one or several intermediate hosts before inoculation in primates.

A sheep scrapie isolate referred to as “Compton”, and serially propagated for 9 passages in goats and then for 8 passages in conventional mice, caused a clinical TSE in Cynomolgus monkey after a 5 years incubation period (intracerebral challenge). When inoculated intracerebrally to Rhesus monkey and Chimpanzee, the same isolate did not transmit at the moment of publication (Gibbs and Gajdusek, 1972). In the same study, the Compton isolate propagated in goats and a field ovine scrapie isolate (from USA) were each inoculated each to a Cynomolgus and a Rhesus monkey. These isolates did not provoke a clinical TSE at the moment when the study was published (4 years post inoculation). The Compton isolate, now propagated for 9 passages in goats, 8 passages in conventional mice and 3 passages in hamster transmitted to squirrel monkey both after intracerebral challenge (incubation period 14 months) (Gibbs and Gajdusek, 1973) or oral challenge (incubation period 25 to 32 months). In this model, sCJD isolates propagated with comparable incubation periods (11-48 months for intracerebral and 23-27 months for oral route) (Gibbs et al., 1980).

These types of experiment remain difficult to interpret since the passage of the original TSE agent into laboratory rodents could have altered its properties.

Results related to Classical scrapie transmission in primates remain very limited, and do not allow evaluating the zoonotic potential of Classical scrapie agents considered in their diversity. There is one study reporting that a natural sheep Classical scrapie isolate (PG 85/02) transmitted to two marmosets after intra-cerebral challenge. The incubation period observed with this scrapie isolate was shorter than with a cattle BSE isolate (Baker et al., 1993).

Conclusions:

- Since the implementation of active surveillance, large numbers of Classical scrapie cases have been identified in most EU Member States.
- Classical scrapie is known to be caused by a range of different TSE agents.
- There is currently no comprehensive picture of the field Classical scrapie strain diversity.
- It can be assumed that, despite SRM measures implemented over the last decade, small ruminants TSE agents have continuously entered into the food chain.

- While transmission data for evaluating the zoonotic potential of Classical scrapie in primates and human PrP transgenic mice are extremely limited or not yet available, a single study reported efficient transmission of a natural sheep Classical scrapie isolate to primates.

4.4. Atypical scrapie

4.4.1. Epidemiology

In 1998 an atypical PrP^{Sc} signature was identified in Norwegian sheep; the partially PK resistant PrP displayed a multi-band pattern in Western Blot that contrasted with those normally observed in small ruminants TSE cases.

A retrospective study carried out in tissues bank allowed to identify Atypical scrapie cases in sheep samples collected in UK in 1987 (Webb et al., 2009). The analysis of data collected through the epidemiosurveillance system in UK between 2002 and 2006 suggested that Atypical scapie prevalence in this population could have remained stable over this period (Green et al., 2008a; McIntyre et al., 2008). Together these elements suggest that Atypical scrapie might have been present in the small ruminant population for several decades.

Since the implementation in 2002 of an active TSE surveillance, similar cases were identified in most EU Members States (Benestad et al., 2003; Moum et al., 2005), and this TSE form was also reported in countries like Canada, USA, and also in New Zealand, which was so far considered to be TSE free.

Atypical scrapie now represents a substantial part of the TSE cases identified in the EU small ruminant population. Some studies described the apparent prevalence of Atypical scrapie in sheep slaughtered for human consumption (healthy slaughtered animals) or collected as fallen stock, based only on tests recommended for the detection of Atypical scrapie on brainstem samples. The apparent prevalence of Atypical scrapie in sheep, although likely underestimated, did not present important variations between countries (Fediaevsky et al., 2008) or over time (Del Rio Vilas et al., 2008; Fediaevsky et al., 2008; McIntyre et al., 2008). Assuming that the monitoring conditions remain comparable in space and time, the distribution of the prevalence of Atypical scrapie appeared relatively homogenous especially compared with the prevalence of Classical scrapie. In a study including 11 European countries that reported Atypical scrapie between 2002 and 2007, the mean prevalence of Atypical scrapie was 5.5 (5.0-6.0) cases per ten thousand in abattoir surveillance, and 8.1 (7.3-9.0) cases per ten thousand in fallen stock (Fediaevsky et al., 2010).

However, there is an important number of caveats to consider in the estimation of the epidemiological situation of Atypical scrapie in Europe.

For instance, the active surveillance system is based on rapid PrP^{Sc} testing in a brainstem sample. However, the brainstem only inconstantly harbours detectable PrP^{Sc} in known positive animals (Benestad et al., 2008). Moreover, not all approved rapid tests are recommended for the detection of Atypical scrapie in brainstem samples (EFSA, 2005b, 2005a). A recent study provided evidence that even CNS containing high infectious titre (as assessed by bioassay in Tg 338 mice) could remain PrP^{Sc} negative using validated screening tests. These results support the notion that a number of Atypical /Nor98 scrapie incubating animals that tested negative could be infected.

It is consequently probable that a certain number of Atypical/Nor98 cases remains undetected, leading to an underestimation of Atypical/Nor98 scrapie incidence in the small ruminant population.

4.4.2. Pathogenesis

Atypical scrapie pathogenesis in its natural host remains poorly documented. Identified Atypical/Nor98 cases are unusually old by comparison to Classical scrapie (Benestad et al., 2008; Buschmann et al., 2004b; Nentwig et al., 2007; Vidal et al., 2008). Data on the Atypical/Nor98 scrapie agent distribution in the organism are limited (Benestad et al., 2003; Buschmann et al., 2004a; Nentwig et al., 2007; Vidal et al., 2007) but seem to indicate, as far as no detectable abnormal PrP has been found in peripheral tissues, that this infectious agent would be restricted to the CNS. These elements were interpreted to support the contention that Atypical/Nor98 scrapie could be a spontaneous disorder of PrP folding and metabolism, occurring in aged animals without external cause (Benestad et al., 2008; Fediaevsky et al., 2010). However no definitive evidence that would support this hypothesis has become available.

While under natural conditions, Classical scrapie is known to transmit between individuals, the analysis of data collected through the active TSE surveillance program seems to indicate that Atypical/Nor98 scrapie could be little or not contagious at all. This statement mainly relies on the lack of statistical difference of the observed Atypical/Nor98 frequencies between the general population and the flocks where a positive case had been identified (Fediaevsky et al., 2010; Fediaevsky et al., 2008). Because of the probably insufficient capacity that we have to detect Atypical scrapie incubating animals using the PrP^{Sc} based methodologies, this contention might be considered with caution.

However, recent information collected in both natural and experimental Atypical scrapie cases seem to indicate that low infectivity levels can be present in skeletal muscle, peripheral nerves and lymphoid tissues of animals incubating or affected with Atypical scrapie (Lacroux et al., 2010).

Emerging data from an oral challenge study indicate that very early oral exposure (within 24 hours of birth) can lead to successful transmission to AHQ homozygous animals after homologous challenge (Simmons et al., 2010b), with clinical disease developing on one animal by 24 months of age.

Several projects are currently ongoing aiming at determining more precisely the pathogenesis of this TSE in the natural host. However considering the nature of the experiment (inoculation in sheep and detection of the agent through bioassay), their definitive results cannot be expected before several years from now.

4.4.3. Transmission *in vivo* and *in vitro*

Transmissibility of Atypical scrapie was demonstrated in both rodent models (transgenic animals expressing the ovine PRP gene) and sheep, confirming that it is associated with an authentic TSE agent (Le Dur et al., 2005; Simmons et al., 2007). To date all available information seems to indicate that Atypical scrapie is likely to be caused by a single TSE agent (Arsac et al., 2007; Griffiths et al., 2010; Le Dur et al., 2005).

Atypical scrapie is a relatively recent discovery and investigations aiming at identifying its capacity to cross transmission barriers are extremely rare. It has recently been reported that an Atypical scrapie isolate was propagated in a model of transgenic mice that overexpressed the Porcine PrP (Espinosa et al., 2009). Primary transmission (intracerebral challenge) occurred in mice with a low attack rate, suggesting a high transmission barrier. The TSE agent that developed shared phenotypic features with the sheep BSE agent (Espinosa et al., 2009). Additional investigations confirmed that the agent that propagated into the porcine PrP transgenic mice model was BSE (Andreoletti et al., 2008).

This result suggests that Atypical scrapie could have the capacity to cross transmission barriers and that during such transmission (in accordance with general principles of TSE diseases) could lead to a radical alteration of its properties.

Similar experiments are currently ongoing in porcine and cattle.

The capacity of Atypical scrapie isolates to propagate in primates and in transgenic mice expressing human PrP is currently under investigation in several laboratories. At the moment of writing no results are available.

Conclusions:

- Atypical scrapie has been present for decades in small ruminant populations. It is probably a disease that is spread worldwide.
- Classical scrapie and Atypical scrapie can exist in the same flock.
- The Atypical scrapie agent harbours particularities that limit our capacity to detect affected animals. It is not possible at the moment to estimate the true disease prevalence.
- Despite SRM measures implemented over the last decade, low levels of Atypical scrapie infectivity might have continued to enter the food chain.
- There are no relevant data available for evaluating the zoonotic potential of Atypical scrapie.

4.5. CWD

4.5.1. Epidemiology

Chronic wasting disease (CWD) is a TSE disorder of unknown etiology, confined mainly to North American mule deer (*Odocoileus hemonius hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*, referred to as elk in the text below) and Shira's moose (*Alces alces shirasi*). It is the only currently recognized TSE disease of wild animals, and also occurs in farmed cervids.

First identified in the late 1960s as a fatal wasting syndrome of mule deer in a northern Colorado research facility (Fort Collins), it was recognized as a TSE disease in 1978 by histopathological assessment (Williams and Young, 1980, 1992). A retrospective assessment also revealed CWD infection of mule deer and black-tailed deer (*Odocoileus hemonius columbianus*) resident at the Toronto Zoo between 1973 and 2003 (Dube et al., 2006). CWD was also identified in mule deer in a research facility in Wyoming and in captive Rocky Mountain elk in both the Colorado and Wyoming facilities, and thereafter in free-ranging mule deer and elk in southeastern Wyoming and northeastern Colorado (Williams and Young, 1980, 1982, 1992). Surveillance and modeling studies indicated that CWD occurred endemically among free-ranging deer and elk in a contiguous area in northeastern Colorado, southeastern Wyoming, and western Nebraska, and that CWD was most likely present in free-ranging cervids in this "endemic region" several decades prior to its eventual recognition (Miller et al., 2000). Most recently CWD has occurred in wild (Baeten et al., 2007) and captive Shira's moose (Kreeger et al., 2006) in the endemic region.

The prevalence of CWD varies across North America, but can be as high as 30% in some areas of Colorado (Williams, 2005). Based on hunter-harvested animal surveillance, the prevalence of CWD in the endemic area from 1996 through 1999 was estimated at approximately 5% in mule deer, 2% in white-tailed deer, and <1% in elk (Spraker et al., 1997). Surveys conducted by the Colorado Division of Wildlife during June 2006 to June 2009 continue to demonstrate wide distribution of CWD in

Colorado. Summaries of harvest survey data varied from <1 to 14.3% among mule deer, <1 to 2.4% among elk, and <1% among moose¹².

The high level of transmissibility combined with extreme deer densities in certain areas of the USA suggests that it will be difficult to control this disease. Indeed, wildlife management efforts to contain or eradicate CWD in Colorado have so far proven unsuccessful (Conner et al., 2007). Long thought to be limited in the wild to the endemic area, since 2002 CWD has also emerged in free-ranging populations of white-tailed deer east of the Mississippi (Joly et al., 2003). CWD has been detected in areas previously thought to be free of infection, including recent discoveries in West Virginia, New York, and Michigan. Currently, CWD has been recognized in wild and/or farm-raised cervids from fifteen North American States and, in addition to its aforementioned detection in Ontario, the Canadian provinces of Saskatchewan and Alberta. While identification in these new areas may be partly related to increased surveillance, spread of the disease by natural migration, and translocation of infected cervids by humans almost certainly plays a role in the emergence of disease. The latter mechanism is exemplified by outbreaks occurring in South Korea as a result of importation of sub-clinically infected animals (Kim et al., 2005; Sohn et al., 2002). During the period 2006-2010 a survey has been carried out in the EU with the aim of detecting the possible presence of CWD and other TSEs in the EU farmed and wild cervid population, with no TSE positive results recorded. Based on the analysis of the results of the survey, a recent EFSA opinion (EFSA Panel on Biological Hazards (BIOHAZ), 2010b) concluded that there is not a cervid TSE epidemic in the EU. However, the same opinion also concluded that the occurrence of cases of TSEs, especially in remote and presently unsampled geographic areas, may not be excluded in cervids in the EU. Prior to this survey, another study was carried out in wild and farmed cervid species in Germany, which did not record any TSE positive result (Schettler et al., 2006). While testing for CWD in other countries has been minimal, limited active surveillance has, to date, not detected CWD in Japan (Kataoka et al., 2005).

4.5.2. Pathogenesis

Clinical features include gradual loss of body condition, and behavioral changes (Williams, 2003). At later stages, affected animals may display polydipsia and polyuria, sialorrhea and generalized incoordination. After diagnosis most animals survive for a few weeks up to 3 to 4 months. Limited transmission studies indicated that CWD developed ~25 % more rapidly in orally challenged elk than deer (16 months for mule deer and 12 months for elk) (Williams, 2003). Maximum incubation periods are not known, but most cases occur in animals 3 to 7 years old, with the majority of animals probably developing CWD within 3 years of infection (Miller et al., 1998).

As demonstrated in other species in which TSE diseases occur naturally, susceptibility to CWD is highly dependent on polymorphic variation in deer and elk *PRNP*. In mule deer, polymorphism at codon 225 encoding serine (S) or phenylalanine (F) influences CWD susceptibility, the 225F allele being protective. The occurrence of CWD was 30-fold higher in deer homozygous for serine at position 225 (225SS) than in heterozygous (225SF) animals; the frequency of 225SF and 225FF genotypes in CWD-negative deer was 9.3%, but only 0.3% in CWD-positive deer (Jewell et al., 2005). Polymorphisms at codons 95 [glutamine (Q) or histidine (H)] (Johnson et al., 2003), 96 [glycine (G) or serine (S)] (Johnson et al., 2003; Raymond et al., 2000) and 116 [alanine (A) or glycine (G)] (Heaton et al., 2003) in white-tailed deer have been reported. While all major genotypes were found in deer with CWD, the Q96, G96, A116 allele (QGA) was more frequently found in CWD-affected deer than the QSA allele (Johnson et al., 2003; O'Rourke et al., 2004). The elk *PRNP* coding sequence is also polymorphic at codon 132 encoding either methionine (M) or leucine (L) (O'Rourke et al., 1998; Schätzl et al., 1997). This position is equivalent to human *PRNP* codon 129. Studies of free-ranging and captive elk with CWD (O'Rourke et al., 1999), as well as oral

¹² http://wildlife.state.co.us/NR/rdonlyres/763F5731-F895-4D52-9F27-2B8D5BE91175/0/CO_CWDreport_06082.pdf

transmission experiments (Hamir et al., 2006a; O'Rourke et al., 2007), indicate that the 132 L allele protects against CWD.

CWD is characterized by extensive CNS and lymphoid tissue deposition of PrP^{Sc}, the latter being detectable early in disease (Fox et al., 2006; Sigurdson et al., 1999). CWD pathogenesis seems to vary between deer and elk with less PrP^{Sc} deposition in lymphoid tissues of elk compared to deer (Race et al., 2007). Also, florid amyloid plaques feature in the neuropathology of diseased deer (Liberski et al., 2001). Other tissues and bodily fluids of deer and elk in which PrP^{Sc} or infectivity has been detected include pancreas (Fox et al., 2006; Sigurdson et al., 2001), adrenal gland (Fox et al., 2006; Sigurdson et al., 2001), and cardiac muscle (Jewell et al., 2006). CWD agents have been detected in saliva and blood by bioassay (Mathiason et al., 2006) and in urine by PMCA (Haley et al., 2009b) and bioassay (Haley et al., 2009a), suggesting a role for these body fluids in transmission and dissemination. Fecal material from subclinical deer also harbors infectivity (Haley et al., 2009a; Tamguney et al., 2009).

4.5.3. Transmission *in vivo* and *in vitro*

CWD is transmissible after intracerebral inoculation of mule deer with incubation periods of up to 2 years (Williams and Young, 1992). The highly efficient transmission of CWD appears unparalleled among TSE diseases (Miller and Williams, 2003; Miller et al., 2000; Williams and Young, 1980). The remarkable transference of CWD is documented in a captive mule deer population wherein 90% of the mule deer present for more than two years ultimately developed disease (Williams and Young, 1980). Although the natural route of CWD transmission is not precisely known, lateral transmission (Williams and Miller, 2002) by ingestion of forage or water contaminated by secretions, excretions, or other sources, for example CWD-infected carcasses (Miller et al., 2004), has long been thought the most plausible natural route.

The generation of CWD-susceptible transgenic mice, and the development of PMCA-based approaches for amplifying CWD infectivity using PrP expressed in the CNS of those mice (Green et al., 2008c; Meyerett et al., 2008) greatly facilitated our understanding of the mechanism of CWD transmission among deer and elk. Prototype transgenic mice expressing deer PrP, designated Tg(CerPrP)1536+/- (Browning et al., 2004), recapitulated the cardinal neuropathological, clinical and biochemical features of CWD, an observation subsequently confirmed in comparable transgenic mouse models expressing deer or elk PrP (Angers et al., 2009; Kong et al., 2005; LaFauci et al., 2006; Meade-White et al., 2007; Tamguney et al., 2006; Trifilo et al., 2007). The presence of CWD agents in saliva, blood, urine, and feces (Haley et al., 2009a; Haley et al., 2009b; Mathiason et al., 2006; Tamguney et al., 2009) is consistent with the aforementioned mechanism of lateral transmission. The detection of CWD agents in elk antler velvet by transgenic bioassay, and the annual shedding of this material raises the possibility that it may also play a role in CWD transmission (Angers et al., 2009).

In addition to its increased geographic spread, the known host-range of CWD is also expanding. Since 2002, CWD has emerged in free-ranging populations of white-tailed deer (Joly et al., 2003). Most recently CWD has occurred in wild (Baeten et al., 2007) and captive Shira's moose (Kreeger et al., 2006), and has been experimentally transmitted to European red deer (*Cervus elaphus elaphus*) (Martin et al., 2009). Whether the host range of CWD extends beyond the family Cervidae is currently unclear. However, the remarkably high rate of CWD agent transmission brings into question the risk posed to livestock from developing a novel CWD-related TSE disease via shared grazing of CWD contaminated rangeland. This issue has been indirectly addressed by transmitting CWD to transgenic mice expressing ovine or bovine PrP, with negative outcomes (Tamguney et al., 2006). Experimental transmission to other species has had mixed results. Studies demonstrated that the CWD agent transmitted poorly to hamsters, ferrets, and mink (Bartz et al., 1998; Marsh et al., 2005; Sigurdson et al., 2008). Non transgenic mice appear resistant to CWD infection (Browning et al., 2004).

The identification and characterization of distinct CWD strains, and the influence of PrP primary structure on their stabilities, is of importance when considering the potential for inter-species transmission. The appearance of vCJD following human exposure to BSE (Bruce et al., 1997; Hill et al., 1997), place the human species barrier to CWD at the forefront of public health concerns. Since North American hunters harvest thousands of deer and elk each year, and it is not currently mandatory to have these animals tested for CWD, the demonstration of CWD agents in skeletal muscle and fat of deer (Angers et al., 2006; Race et al., 2009a), makes it likely that humans consume CWD agents. The substantial market for elk antler velvet in traditional Asian medicine also warrants concern (Angers et al., 2009). Surveillance currently shows no evidence of CWD transmission to humans (Belay et al., 2004; Mawhinney et al., 2006). Moreover, CWD agents have hitherto reassuringly failed to induce disease in transgenic mice expressing human PrP (Kong et al., 2005; Sandberg et al., 2010; Tamguney et al., 2006). Systematically addressing the zoonotic potential, as well as the tissue distributions of CWD1 and CWD2 strains in infected deer and elk, would nonetheless appear to remain high priorities. Determining the levels of infectivity in different tissues of infected cervids is also an important component of risk assessment. The development of CWD susceptible transgenic mice (Angers et al., 2009; Browning et al., 2004) and, more recently, CWD-susceptible cell lines (Bian et al., 2010) have allowed the quantification of CWD infectivity by endpoint titration and by cervid prion cell assay respectively.

CWD was successfully transmitted to squirrel monkeys (Marsh et al., 2005; Race et al., 2009b), but macaques remained healthy 70 months post-inoculation of CWD-infected brain by the intra-cerebral route, suggesting a lower, if any, virulence of this agent in macaque (Race et al., 2009b). Further experiments are still ongoing in several laboratories to assess the zoonotic potential of different CWD strains in primate models.

Conclusions:

- CWD has not been reported in the EU.
- Although CWD agents have failed to induce disease in transgenic mice expressing human PrP, experimental transmission to certain non-human primate species has been reported.

4.6. TME

TME is an episodic TSE disease, but is peculiar since it is the only animal TSE disease known so far that is affecting carnivores, while all the other ones affect ruminants under natural conditions. The zoonotic risk of this disease seems to be low and may be rather considered as an epidemiological cul-de-sac, but the context of this disease is rich of information for inter-species transmission purposes.

4.6.1. Epidemiology

TME is a disease that affected mink ranches, decimating the herds, in the frame of five to eleven isolated outbreaks, from 1947 to 1985, mainly in USA (Wisconsin was the main affected State since it is where mink ranches were mainly located) (Robinson et al., 1994). Moreover, eastern European countries (East Germany, Finland and USSR) also reported outbreaks in the mid-sixties.

According to its rare occurrence associated with a massive rate of infection, and in the absence of probing horizontal or vertical transmission, the hypothesis of a food-borne infection is the most convincing explanation. This theory is enforced by the fact that three of those outbreaks occurred in large mink production facilities that prepared on-site feed involving the use of non-ambulatory (i.e. animal unable to stand alone) cattle (Hartsoug and Burger, 1965; Marsh et al., 1991).

4.6.2. Pathogenesis

Several experiments were set up to elucidate the origin of the TME agent. Experimental inoculation of minks with scrapie (Marsh and Hadlow, 1992) or CWD (Harrington et al., 2008) isolates led to incomplete rates of transmission of neurological diseases, in which features differed from those of the natural TME. Inoculation of Classical BSE led to a complete transmission of the disease with shorter incubation periods (12 months), but the induced disease was also different from original TME (Robinson et al., 1994).

Reverse experiments were also performed, notably in cattle (Hamir et al., 2006b; Robinson et al., 1995): the intra-cerebral inoculation of TME isolates to cattle induced a TSE, but that differed from Classical BSE. At the time of those experiments, Atypical BSE was unknown, and comparisons of this experimentally-induced cattle TSE with those latter has not been documented until now.

4.6.3. Transmission *in vivo* and *in vitro*

Few experiments were set up for assessing zoonotic risk of TME for human health, since TME outbreaks are rare and no case was reported since 1985. Moreover, risks of transmission may be only limited to accidental professional exposure, since there is no risk of foodborne transmission of TME from mink to human.

Nevertheless, transmission experiments to squirrel monkeys were successful, inducing a disease in 10 to 14 months (Eckroade et al., 1970). In parallel, transmissibility to cynomolgus macaque was assessed by inoculation of TME isolate not through intracerebral route, but through various peripheral routes (subcutaneous, intra-muscular, intravenous, oral) (Marsh et al., 1969). Thirty-three months post-inoculation, the primate remained healthy, but its organs, including brain and spleen, showed infectivity through reverse transmission to mink.

TME was successfully transmitted to cattle (Hamir et al., 2006b), and it was proposed that the resulting cattle TSE disease might correspond to a natural cattle TSE agent at the origin of (at least some) TME outbreaks. In this context, the transmissibility of this cattle TSE agent to human was assessed in a macaque model: transmission was successful, inducing a disease in macaque very similar to the one induced by inoculation of the L-BSE isolate (Comoy et al., 2009). The similarity of TME and L-BSE strains has also be highlighted in a transgenic model of mice overexpressing ovine PrP (Baron et al., 2007).

Conclusions:

- Risk of human exposure to TME is remote (historical rare occurrence, no dietary exposure, the risk is limited to accidental professional exposure).
- Properties of the TME agent are similar to those of L-BSE, including the capacity to propagate efficiently in primates.
- There is some evidence to indicate that the TME agent has the potential to be a zoonotic agent.

4.7. Human TSE diseases

4.7.1. Epidemiology

4.7.1.1. Sporadic CJD

Evidence that sCJD was caused by a transmissible agent was published in 1968 (Gibbs et al., 1968) but a series of epidemiological studies, in a number of different countries, have failed to identify an environmental source of infection. Furthermore sCJD occurs worldwide with a relatively stable incidence, including countries which are thought to be free of animal TSEs such as scrapie. Mortality rates for sCJD are relatively uniform at 1-2 cases per million population per annum in countries with established surveillance systems and the overall mortality rates in countries collaborating in the EuroCJD system are listed in Table 4.

sCJD is phenotypically heterogeneous and this is related in part to variation in genetic background and the biochemical form of prion protein deposited in the brain. The wide variation in clinical and pathological characteristics in sCJD makes identification of novel forms of human TSE disease difficult and continuing careful systematic study of CJD in populations is necessary to identify new phenotypic subtypes.

4.7.1.2. Variant CJD

This is a novel disease, identified in 1996, and there is compelling evidence that it is caused by the transmission of BSE to humans, probably through the past consumption of high titre bovine tissues in the human food chain. The major risk factors for vCJD are a young age, methionine homozygosity at codon 129 of *PRNP* and residence in the UK.

The number of cases of vCJD identified worldwide as of July 2010 are shown in Table 3.

Table 3: Variant CJD, current data (updated to July 2010).

Country	Total number of primary cases (number alive)	Total number of secondary cases: blood transfusion (number alive)	Residence in UK > 6 months during period 1980-1996
UK	170 (4)	3 (0)	173
France	25 (0)	-	1
R of Ireland	4 (0)	-	2
Italy	2 (1)	-	0
USA	3† (0)	-	2
Canada	1 (0)	-	1
Saudi Arabia	1 (0)	-	0
Japan	1* (0)	-	0
Netherlands	3 (0)	-	0
Portugal	2 (0)	-	0
Spain	5 (0)	-	0

† The third US patient with vCJD was born and raised in Saudi Arabia and has lived permanently in the United States since late 2005. According to the US case report, the patient was most likely infected as a child when living in Saudi Arabia.

* The case from Japan had resided in the UK for 24 days in the period 1980-1996.

Table 4: Sporadic CJD: annual mortality rates per million (data for 2010 are incomplete).

	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	Mean Rate
Australia	0.96	0.62	1.05	1.37	1.08	1.34	1.32	1.46	1.03	0.92	1.11	0.84	1.18	1.59	1.23	1.35	0.82	0.41	1.09
Austria	0.77	1.15	1.15	1.15	0.77	1.03	0.77	1.12	1.12	0.87	1.86	0.99	1.74	2.23	2.23	0.99	1.98	0.62	1.29
Belgium					0.97	1.46	1.26	0.87	1.55	2.14	1.94	1.94	1.17	1.46	1.65	0.28	1.12	n/a	1.37
Canada		0.07	0.1	0.44	0.53	0.73	0.86	1.04	0.87	0.99	0.85	1.31	1.30	1.20	0.97	1.26	1.30	0.12	1.01
Cyprus			1.48	0	0	0	0	0	1.43	0.00	1.39	1.37	0.00	1.30	1.28	3.84	0.00	0	0.78
Czech Republic								0.19	0.78	0.59	0.78	1.08	0.98	0.97	1.07	1.05	1.43	0.57	0.90
Denmark					2.08	0.94	1.32	0.94	1.12	1.51	1.89	1.13	0.57	1.51	1.10	1.09	1.25	n/a	1.27
Estonia												0	0.74	0	0	0	0	0.75	0.21
Finland					1.92	0.96	1.35	0.77	1.73	1.92	0.96	1.15	2.5	0.77	0.72	2.5	1.13	n/a	1.41
France	0.6	0.8	1.02	1.18	1.38	1.38	1.53	1.46	1.79	1.76	1.76	1.56	1.3	1.97	2.17	1.63	1.68	0.54	1.42
Germany	0.44	0.85	1	0.93	1.3	1.4	1.25	1.36	1.53	1.14	1.2	1.3	1.2	1.29	1.24	1.28	0.8	n/a	1.15
Greece					0.55	0.37	0.64	0.83	0.73	1	0.55	0.64	0.46	0.55	0.63	0.54	n/a	n/a	0.62
Hungary					1.10	0.30	0.50	0.90	1.70	0.70	0.80	0.80	0.80	1.20	1.30	1.40	0.70	0.5	0.91
Iceland					3.7	0	0	0	0	0	0	0	0	0	3.23	0	0	n/a	0.53
Ireland				0.55	0.55	1.62	0.27	0.79	1.30	1.28	0.50	1.73	1.21	0.94	0.71	1.18	0.47	0.24	0.92
Israel	0.37	0.19	0.56	0.49	0.66	0.82	0.49	0.49	0.6	0.45	1.2	1.2	0.9	0.68	0.68	1.1	0.82	0.13	0.68
Italy	0.48	0.58	0.49	0.89	0.83	1.11	1.35	1.04	1.47	1.35	1.37	1.35	1.87	1.66	1.66	1.57	1.75	1	1.21
Latvia										0	0	0	0	0	0.43	0.43	0	0.89	0.12
Netherlands	0.79	1.17	0.52	0.9	1.2	1.13	1.24	0.63	0.88	1.13	0.75	1.22	1.22	1.34	0.92	0.98	0.66	1.56	0.98
Norway					1.33	0.44	0.44	0.67	1.11	0.44	1.33	1.56	0.89	1.08	1.11	0.86	1.04	0	0.95
Poland										0.16	0.31	0.52	0.36	0.13	0.08	0.26	0.86	n/a	0.34
Portugal					0.50	0.60	0.50	0.50	1.60	0.60	1.10	0.60	1.00	0.50	0.80	0.40	0.56	0.19	0.70
Slovakia	0.4	0.4	0.4	0.4	0.6	0.6	0.2	0.4	0.4	1.13	0.4	1.17	1.34	0.57	0.75	1.13	0.75	0.37	0.65
Slovenia	0	0.5	1.0	0	0	0.50	1.51	0.50	2.01	2.51	1.00	1.00	1.00	3.49	0.99	1.46	0.99	0	1.12
Spain	0.54	0.41	0.51	0.69	0.79	1.57	1.27	1.14	1.57	1.36	1.44	1.36	1.79	1.44	1.65	1.30	1.19	0.65	1.17
Sweden					1.13	1.58	1.24	1.58	1.35	1.34	1.23	1.66	1.44	1.76	1.20	1.40	1.18	n/a	1.41
Switzerland	1.15	1.42	1.42	1.41	1.41	1.12	1.26	1.53	2.62	2.46	2.31	2.16	1.34	1.73	2.11	1.81	2.4	1.04	1.7
UK	0.62	0.93	0.6	0.69	1.03	1.09	1.06	0.85	0.98	1.21	1.33	0.84	1.1	1.14	1.03	1.42	1.26	0.87	1.01

n/a: not available

The incubation period in vCJD has been estimated from epidemiological data and from mathematical modelling to have a mean of about 15 years, although with a significant spread of incubation periods around this figure. The minimum incubation period in iatrogenic CJD (after peripheral inoculation) and kuru is around 5 years and the maximum in kuru may extend beyond 4 decades. Therefore the incubation time of a new TSE would be impossible to predict. The assessment of any possible link between animal and human TSEs must take into account the potential for prolonged incubation periods, particularly across a species barrier, with the implication that the period of observation must be taken into consideration when assessing potential links. In atypical animal TSEs it is not known whether these are novel diseases or only newly recognised and it may be necessary to extend the period of observation before any possible links between these diseases and human TSEs can be excluded.

To date, all tested cases of definite or probable vCJD have had a specific genetic background, methionine homozygosity at codon 129 of the prion protein gene. In December 2009 a UK case of possible vCJD with a heterozygous background was published raising the possibility that individuals with this genotype may be susceptible to BSE infection and that a further outbreak of vCJD is possible. Previous mathematical modelling has suggested a relatively limited outbreak of cases of vCJD should this genotype confer susceptibility to BSE infection and that the number of cases in such a scenario would be unlikely to exceed the primary outbreak. A further issue is that there may be a population of individuals who are sub- or preclinically infected with the vCJD agent and some may not develop clinical symptoms within their lifespan; there have been several attempts at estimating the prevalence of vCJD with the UK population by screening lympho-reticular tissue for abnormal prion protein by immunohistochemistry and related methods (Clewley et al., 2009; de Marco et al., 2010; Hilton et al., 2004) and a new initiative which will involve anonymous screening of about 40,000 appendix samples has recently begun¹³.

4.7.1.3. Genetic human TSE disease

These forms of disease, including genetic CJD, Gerstmann-Sträussler-Scheinker disease and Fatal Familial Insomnia are dominantly inherited and associated with mutations of the human prion protein gene. The favoured hypothesis is that the mutation of the prion protein gene results in an increased chance of conversion of normal prion protein to the disease associated form and that these types of human TSE disease are not environmentally acquired. Cases of genetic human TSE disease have been identified in many countries and, although there is variation in incidence by country, overall the incidence rate is about one tenth that of sCJD.

4.7.1.4. Proteinase sensitive prionopathy

This rare disorder was recently identified (Gambetti et al., 2008) and is not yet known to be experimentally transmissible and may not be an “infectious” TSE disease. It is associated with valine homozygosity in the great majority of cases and about half the cases have a family history of a neurodegenerative disorder.

4.7.1.5. Iatrogenic CJD

This acquired or “infectious” form results from human-to-human transmission, in the vast majority of cases by dura mater transplants or treatment with cadaveric pituitary hormone extracts. All genotypes at *PRNP* codon 129 have been shown as susceptible, although heterozygosity lengthens the incubation period and seems to be protective (Deslys et al., 1998).

¹³ Source: Health Protection Agency, available at <http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/CreutzfeldtJakobDisease/AbnormalPrionProteinPrevalenceStudies>

4.7.1.6. Kuru

Kuru is a historical disease caused by ritual cannibalism and is restricted to certain regions of Papua New Guinea. The disease has virtually disappeared but cases with incubation periods exceeding four decades have been identified.

4.7.2. Pathogenesis

In most forms of human TSEs infectivity is largely restricted to the CNS. In vCJD there is more widespread distribution of infectivity particularly in the lymphoreticular system.

Iatrogenic CJD is caused by transmission of sCJD from person to person in the course of medical treatment. There have been more than 400 cases of iatrogenic CJD worldwide, the majority related to human pituitary growth hormone and human dura mater grafts. The number of cases of iatrogenic CJD is in decline.

There is compelling evidence that vCJD is transmissible from person to person through blood transfusion and it is possible that vCJD has been transmitted iatrogenically through the plasma product, Factor VIII, used in the treatment of haemophilia. The potential for TSEs to be transmitted by secondary infection increases the importance of a careful assessment of potential risk factors for human disease.

4.7.3. Transmission *in vivo* and *in vitro*

Laboratory transmission studies have been carried out extensively in human TSE diseases. In sCJD such studies had mainly been carried out in primates, because wild type mice are largely resistant to infection. These studies have confirmed that sCJD is experimentally transmissible (Beck et al., 1969b; Gibbs et al., 1968). Recently transmission studies have been carried out in transgenic models and have indicated strain diversity in sporadic CJD.

Laboratory transmission studies in vCJD have confirmed the similarity of the infectious agent in vCJD and BSE.

There must be some uncertainty about the relevance to natural disease of evidence of potential cross-species transmission in artificial laboratory experiments.

Conclusions:

- Human TSEs occur in sporadic, genetic and acquired forms.
- There is limited information on the biological diversity of TSE agents in humans.
- Detected human sporadic cases are rare with mortality rates of 1-2 cases per million population per annum. This rarity and the necessarily passive surveillance system limits epidemiological analysis.
- vCJD was predominantly caused by dietary exposure to the Classical BSE agent.
- The vCJD epidemic is in decline, but there are concerns that there may be further outbreaks related to variation in the codon 129 polymorphism or through secondary transmission.
- Current measures to minimise human exposure to animal TSEs have been demonstrated to be effective against Classical BSE.

5. Uncertainties

A guidance of the EFSA Scientific Committee (EFSA, 2009a) concluded that:

- *“There may be differences in risk due to variability among individuals, populations, species or ecosystems. It is important to identify and describe the most influential contributors to variability in risk, preferably by statistical analysis of the underlying data. Any statistical difference must be interpreted in the light of its biological relevance.”*
- *“Although it may be impossible to identify all the uncertainties, the assessment should include a description of the types of uncertainties encountered and considered during the different risk assessment steps. Their relative importance and their influence on the assessment outcome should be described.”*

There are considerable uncertainties in our understanding of TSEs and their ranking of importance to the risk assessment process is a source of debate. The following could be considered amongst others in order of relative importance to this risk assessment:

1. The uncertainty on “the nature of the infectious agent”.

There are a range of views. Of course it would be intellectually more satisfying to know exactly what the infectious agent is at the level of its molecular architecture and accessory molecules (lipids, nucleic acids or carbohydrate). Even without this knowledge, it can be accepted, based on the results from *de novo* generation and other *in vitro* conversion experiments, and transmission experiments to human PrP transgenic mice, that the human prion protein can be converted to a PrP^{Sc}-like form by animal PrP^{Sc}: there is not an absolute barrier to infection/conversion of humans by mammalian TSEs at the molecular level.

This is an important insight: its implication is that, irrespective of the precise nature of the infectious agent, there are sufficient data to say that animal prions have a potential to infect humans.

2. The uncertainty on “the global distribution of animal and human TSEs”.

The current level of surveillance for animal and human TSEs using current methodologies and case definitions has led to reported annual mortality rates of human TSE of about 1-2 in 10⁶ total population worldwide, and high variability in the incidence/prevalence of animal TSEs in different countries and continents. Sub-categorisation of the types of animal and human TSEs may refine the subtleties of the epidemiological analyses but increases the uncertainty of these estimates. In the specific case of vCJD, the long-term evolution of the epidemic in non-MM genotypes, the influence of non-PRNP genetic factors and the clinical variation due to different exposure routes (blood transfusion, plasma-derived products etc.) are difficult to predict and may contribute to an under-estimation of the prevalence of infection with vCJD.

Irrespective of this uncertainty, the incidence of human TSEs as currently defined for epidemiological purposes is very low, and consequently the risk of developing disease by transmission of animal prions to humans must be also correspondingly very low. However, the real prevalence of infection (in contrast to disease) in humans due to exposure to BSE or other animal TSEs is largely unknown, even for vCJD/BSE.

3. The uncertainty on “the relative efficiencies of transmission between and within species”.

In past risk assessments, this effective barrier to transmission between species has been set as 1 (no barrier) for cattle BSE to humans as a worst case, or effectively “infinity” (no transmission) for small ruminant TSEs to humans. Various likely estimates will lie somewhere between these

two extremes. This is a key parameter for quantifying human risk of contracting an animal TSE and, while it is effectively unknown, it should be seen in the context of the low overall chance of developing a known type of human TSE of whatever aetiology (annual rate of about 1-2 in 10^6 total population).

Irrespective of this uncertainty, the incidence of human TSEs as currently defined for epidemiological purposes is very low, and consequently the risk of transmission of animal prions to humans must be correspondingly very low. However, the real prevalence of infection (in contrast to disease) in humans due to exposure to BSE or other animal TSEs is largely unknown, even for vCJD/BSE.

4. The uncertainty on “the degree of confidence in case recognition in the situation where not all aspects of the case definition are met”.

This covers a range of clinical, neuropathological and biochemical criteria for diagnosis of a case of TSE in animals and humans. Normally, these methodologies have been assessed for their individual diagnostic sensitivity and specificity and they are used in combination to re-inforce and increase confidence in the diagnosis. These parameters are usually incorporated into an epidemiological analysis and are also discussed in Chapter 3 of this Opinion (“Tools and methodologies”).

This uncertainty can be quantified and should be. However, it is unlikely to contribute to a significant increase in the estimated prevalence of human disease.

5. The uncertainty on “the origin of BSE”.

The type and origin of the (“scrapie-like”, L-BSE or H-BSE) prions that initially caused cases of BSE in cattle are unknown, and unlikely to be elucidated, but the identification of MBM (and possibly other by-products, tallow etc.) in animal feed as the “extended” common source of the epidemic is an epidemiological fact.

This uncertainty can be ignored for the purposes of this risk assessment.

6. The uncertainty on “the origin of sCJD”.

There is speculation that sCJD is due to exposure to Atypical BSE, or is linked to Classical scrapie, Atypical scrapie etc.

This uncertainty is the subject of this risk assessment.

7. The uncertainty on “why procedures that normally denature proteins can only reduce but not totally eliminate infectivity”.

This is part of the generic uncertainty surrounding “the nature of the infectious agent” (See Point 1 above). It is possible that failure to inactivate prions may facilitate their transmission to humans and animals. In this case, it could be argued that “resistant” prions are more likely to transmit to humans than “weak” prions and knowledge of the relative resistance of animal prions could help rank different animal TSEs as potential zoonotic agents. This is speculative, and was originally suggested to explain the increased BSE risk of MBM following the change in rendering procedures at the end of the 1970s but this did not stand up to experimental verification. The Atypical BSE and scrapie agents are currently being tested for their resistance to standard autoclave procedures.

This uncertainty has less importance for the purposes of this risk assessment, but has to be considered in the frame of exposure risk and possible transmission risk from animal products

including medicines to humans with respect to the difficulties to eliminate prions when the source material is contaminated.

8. The uncertainty on “the function of the PrP^C protein”.

Comprehensive knowledge of the (probable) multi-functional role of PrP would help understand the possible range of prion-protein-related diseases, some of which might not be transmissible and some of which might not have accumulation of an aggregated form as a pathological consequence.

This uncertainty can be considered as irrelevant in the framework of this risk assessment.

9. The generic uncertainty surrounding “the presence of zoonotic TSEs in small ruminants”.

More specifically, there are uncertainties about the prevalence of BSE in small ruminants in the EU/world, and the uncertainties of “unconfirmed cases of BSE in goats”¹⁴ in individual Member States, and our lack of ability to define *a priori* zoonotic and non-zoonotic strains individually or in a mixed infection. In past risk assessments, in the absence of evidence to the contrary, TSEs (apart from BSE) in small ruminants have been considered to be due to a non-zoonotic pathogen (See Point 2 above).

Every practical effort should be made to elucidate the zoonotic potential of various small ruminant TSE strains, so their prevalence can be monitored against the background of benign strains. However, in the absence of evidence to the contrary, TSE agents (apart from the Classical BSE agent) in small ruminants can not be considered to be zoonotic pathogens (See Point 2 above).

10. The uncertainty on “whether prions are retained with or without replication in the digestive and other tissues of non-mammalian species fed feedstuffs containing mammalian prions”.

- The replication of mammalian prions in birds or fish has not been observed in the limited number of challenge studies which have been done in animals of these non-mammalian phyla.
- While sequence identity itself is not a good correlate of cross-species transmission, the lack of major amino-acid sequence similarity between their prion or prion-like proteins, reduces considerably the likelihood of amplifying the level of prions by feeding to birds or fish.
- The persistence of prions in fish and birds in a non-replicative state following their exposure is possible and this persistence is likely to be similar or less than their persistence in association with plants or inorganics. The level of persistence of prions in soils has been investigated and could provide some quantification of this uncertainty.
- The low likelihood of cattle or small ruminant prion amplification and persistence in non-mammalian species is not quantifiable, although, if it did occur, these species could represent a secondary vector of exposure between humans and animals confounding attempts to establish an epidemiological link between human and ruminant TSEs.

This uncertainty can be discounted in this overall risk assessment on the zoonotic potential of TSEs, but has to be considered when addressing human exposure to animal prions and the possibilities for crossing the human-animal species barrier (See Point 3 above).

¹⁴ A case of caprine TSE diagnosed in 1990 in a Scottish goat was identified retrospectively by PrP IHC to have characteristics usually seen in small ruminant BSE cases (Jeffrey et al., 2006).

6. Synopsis

The primary objective of this document is to assess potential associations between animal and human TSEs. An assessment of the potential causal links between animal and human TSEs according to the Bradford Hill's guidelines (Bradford Hill, 1965) is shown in Table 5 below. One conclusion is that only the BSE/vCJD link is established. It is important to stress that future scientific information might result in changes to the assessment of one or more of the other animal TSEs.

Table 5: Assessment of Putative Links Between Animal and Human TSEs according to the criteria of the Bradford Hill guidelines.

Criteria	Cattle BSE	Small ruminant BSE ^(a)	Atypical BSE (L-BSE)	Atypical BSE (H-BSE)	CWD	Classical scrapie ^(b)	Atypical scrapie
1. Strength	+						
2. Consistency	+						
3. Specificity	+						
4. Temporality	+						
5. Biological gradient	+						
6. Plausibility	+	+	+	+	+	+	+
7. Coherence	+	+/-					
8. Experiment	+	+	+		+/-	+/- ^(c)	
9. Analogy	+	+	+	+	+	+	+

+: some scientific evidence is available for a positive interpretation

+/-: debatable or conflicting evidence is available

^(a): Classical BSE has not been identified in sheep, but two cases of BSE in goat have been reported in France and UK

^(b): there are multiple strains of the Classical scrapie agent

^(c): a single study has reported transmission of a natural sheep Classical scrapie isolate to primates

As detailed in the document, several tools are available to diagnose and confirm the presence of a TSE, to identify presence or absence of infectivity and potentially measure infectious titres, to characterise the TSE agents infecting an animal or human, and to assess the potential for TSE agents to cross a species barrier. The available methodologies range from epidemiology to *in vivo* and *in vitro* methods. In most instances a combination of tools is required to address specific issues relating to the inter-species transmission potential of TSEs. In humans, neuropathological examination still remains one of the most important tools for a definite diagnosis of TSEs and for the recognition of novel phenotypes. Two complimentary approaches can be used to assess the zoonotic properties of animal TSE agents in transmission experiments: the inoculation of relevant model(s) of the human transmission, and the comparison of disease phenotype of human and animal TSE isolates after their propagation in same animal models, so as to identify the involvement of a potentially common TSE agent. Currently, human PrP transgenic mice and primates are the most relevant models of the human transmission barrier. Because of the potential impact of interspecies transmission on TSE agent properties (e.g. "convergence" phenomenon), the results of comparative study of human and animals TSE isolates after their transmission into a common host species should be considered with caution. A way to circumvent this would be to compare their behavior in several experimental models, if possible.

Biochemical properties of human and animal PrP^{Sc} can be used as part of a classification system for disease phenotypes within a particular species. Similar or even identical PrP^{Sc} biochemical signatures

in different cases within the same or in different species are not interpretable in isolation as proof of infection with the same TSE agent. The qualitative correlation between *in vivo* data and *in vitro* results suggests that *in vitro* conversion assays may be developed as a tool for quantifying the transmission barriers between diverse species and for different TSE strains. So far, most TSE agents propagated through PMCA retain the biological specificities of the original agents. However, there is at the moment no means by which to calibrate and transpose the ease of heterologous conversion *in vitro* into the likelihood of transmission between species *in vivo*.

CONCLUSIONS

CONCLUSIONS

- At present, the only TSE agent demonstrated to be zoonotic is the Classical BSE agent.
- In general, detected cases of sporadic CJD are randomly distributed in time and geographical location. These observations have been interpreted as a supportive argument that sporadic CJD is not environmentally acquired. However, the epidemiological evidence in relation to sporadic CJD cannot be regarded as definitive, and the possibility that a small proportion of cases are zoonotic cannot be excluded.
- Except for Classical BSE, there is limited epidemiological information on the prevalence and distribution of individual ruminant TSE agents.
- These uncertainties indicate that even a rough comparison of the present epidemiological patterns of human TSEs and animal TSEs other than Classical BSE is unlikely to be informative. Thus, it is an imperative to continue to carry out systematic surveillance of human TSEs, and to continue and improve the surveillance of animal TSEs.
- The active screening has allowed the identification of three new forms of animal TSEs (L-type Atypical BSE, H-type Atypical BSE, Atypical scrapie). However, the information obtained has major limitations due to the unknown sensitivity of the monitoring system for these TSEs.
- There is no epidemiological evidence provided to suggest that Classical scrapie is zoonotic.
- The epidemiological data are too limited to conclude whether the Atypical scrapie agent has a zoonotic potential.
- Transmission experiments to human PrP transgenic mice suggest that some TSE agents other than the Classical BSE agent in cattle (namely L-type Atypical BSE and Classical BSE in sheep agents) might have zoonotic potential, whereas for other agents there is no evidence provided of zoonotic potential (H-type Atypical BSE and CWD agents) or no published studies are available (Classical and Atypical scrapie agents).
- Transmission experiments to primates suggest that some TSE agents other than the Classical BSE agent in cattle (namely L-type Atypical BSE, Classical BSE in sheep, TME, CWD agents) might have zoonotic potential. In particular, primates are highly permissive to L-type Atypical BSE, even by the oral route.
- Laboratory transmission experiments indicate that the L-type Atypical BSE agent has a significant zoonotic potential, which appears similar or even higher than that of the Classical BSE agent.
- While transmission data for evaluating the zoonotic potential of Classical scrapie in primates and human PrP transgenic mice are extremely limited or not yet available, a single study reported efficient transmission of a natural sheep Classical scrapie isolate to primates.
- It is unpredictable whether a TSE agent will transmit to a new host and, if the transmission principally occurs, what the transmission rate will be.
- Human PrP transgenic mice and primates are the most relevant models for investigating the human transmission barrier. To which extent such models are informative for measuring the zoonotic potential of an animal TSE under field exposure conditions is unknown. The Classical

BSE agent, as known zoonotic agent, might be used as a benchmark to evaluate the zoonotic potential of other animal TSE agents.

- The ability to create TSE agents by *in vitro* conversion assays with a novel or unprecedented host range (such as those that can infect rabbits) indicate that there is probably no absolute molecular barrier to transmission of TSE agents between mammalian species.
- The qualitative correlation between *in vivo* data and *in vitro* results suggests that *in vitro* conversion assays may be developed as a tool for quantifying the transmission barriers between diverse species and for different TSE agents. However, there is at the moment no means by which to calibrate and transpose the ease of heterologous conversion *in vitro* into the likelihood of transmission between species *in vivo*.

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APPENDICES

A. WILD TYPE AND TRANSGENIC MICE MODELS

Table 1: Strain typing panel in wild type mice.

Strain of mouse	<i>PRNP</i> allele	References
C57Bl(Dk)	a	Dickinson et al., 1968; Dickinson, 1976; Bruce et al., 1991
VM	b	Dickinson et al., 1968; Dickinson, 1976; Bruce et al., 1991
C57Bl X VM (F ₁ cross)	ab	Dickinson et al., 1968; Dickinson, 1976; Bruce et al., 1991
R111	a	Dickinson et al., 1968; Dickinson, 1976; Bruce et al., 1991

Table 2: Gene targeted transgenic lines.

Expressed PrP	Designation of transgenic line	Transgene	Expression level	References
mouse	129/Ola PrP A	MoPrP-A	1	Moore et al., 1998
mouse	129/Ola PrP gtB	MoPrP-B	1	Moore et al., 1998
human	HuM	HuPrPM	1	Bishop et al., 2006
human	HuV	HuPrPM	1	Bishop et al., 2006

Table 3: Standard transgenic lines.

Expressed PrP	Designation of transgenic line	Transgene	Transgenic Vector	Expression level	References
Mouse	Tga20	MoPrP-A	PhgPrP	10	Fisher et al., 1996
Mouse	TG 94	MoPrP-B	CosSha.Tet	4	Hsiao et al., 1990
Mouse	TG 117	MoPrP-B	CosSha.Tet	4	Hsiao et al., 1990
Mouse	Tg4053/FVB	MoPrP-A	CosSha.Tet	8	Carlson et al., 1994
Mouse	Tg46 MloxP	MloxP Prnp a	CosSha.Tet	1	Mallucci et al., 2002, 2003
Mouse	Tg37 MloxP	MloxP Prnp a	CosSha.Tet	3	Mallucci et al., 2002, 2003
Hamster	Tg7	HaPrP	cosHaPrP	1	Hsiao et al., 1990
Hamster	Tg10	HaPrP	cosSha.Tet	Nd	Race et al., 1995
Hamster	Tg81	HaPrP	cosHaPrP	1	Scott et al., 1989
Hamster	Tg71	HaPrP	cosHaPrP	1	Scott et al., 1989
Hamster	Tg52NSE	HaPrP	NSE-promotor	Nd	Race et al., 1995
Human	Tg152	HuPrPV	cosSHaTet	4-8	Telling et al., 1995
Human	Tg650	HuPrPM	human DNA PAC	5-6	Beringue et al., 2007, 2008
Human	Tg110	HuPrPV	cosSHaTet	4-8	Telling et al., 1994
Human	Tg440	HuPrPM	cosSHaTet	2	Telling et al., 1995
Human	Tg35	HuPrPM	cosSHaTet	4-8	Asante et al., 2002
Human	Tg45	HuPrPM	cosSHaTet	4-8	Asante et al., 2002
Human	Tg1	HuPrPM	phgPrP	2	Kong et al., 2005
Human	Tg40	HuPrPM	phgPrp	1	Kong et al., 2005
Sheep/Goat	TgshpXI	OvPrP ARQ allele	phgPrP	2-4	Kupfer et al., 2007
Sheep/Goat	TgshpIX	OvPrP ARQ allele	phgPrP	4-8	Kupfer et al., 2007

Expressed PrP	Designation of transgenic line	Transgene	Transgenic Vector	Expression level	References
Sheep/Goat	Tg301	OvPrP VRQ allele	sheep DNA BAC	8	Vilotte et al., 2001
Sheep/Goat	Tg335	OvPrP VRQ allele	BAC insert	1	Vilotte et al., 2001
Sheep/Goat	Tg338	OvPrP VRQ allele	sheep DNA BAC	8	Laude et al., 2002; Le Dur et al., 2005; Langevin et al., 2011
Sheep/Goat	Tg(ShePrP) 1855	OvPrP ARQ allele	cosSha.Tet		Westaway et al., 1994
Sheep/Goat	Tg(ShePrP) 83669	OvPrP ARQ allele	cosSHa.Tet		Westaway et al., 1994
Sheep/Goat	TgovPrP4	OvPrP ARQ allele	NSE-promotor	3	Crozet et al., 2001
Sheep/Goat	TgovPrP59	OvPrP ARQ allele	NSE-promotor	3	Cordier et al., 2006
Pig	PoTg001	Porcine PrP	phgPrP	4	Castilla et al., 2004a
Pig	PoTg027	Porcine PrP	phgPrP	16	Castilla et al., 2004a
Cow	TgbovXV	bovine PrP (6 OR)	phgPrP	8	Buschmann et al., 2000
Cow	BoPrP4125	bovine PrP (6 OR)	cosSHa.Tet	8-16	Scott et al., 1997
Cow	BoPrP4092	bovine PrP (6 OR)	cosSHa.Tet	8-16	Scott et al., 1997
Cow	tg540	bovine PrP (6 OR)	CMV promotor	8-10	Beringue et al., 2006
Cow	boTg110	bovine PrP (6 OR)	phgPrP	8	Castilla et al., 2003
Cow	boTg035	bovine PrP (6 OR)	phgPrP	6	Castilla et al., 2003
Cow	boTg078	bovine PrP (6 OR)	phgPrP	2	Castilla et al., 2003
Cow	boTg022	bovine PrP (6 OR)	phgPrP	0.5	Brun et al., 2007
Cow	boTg004	bovine PrP (5 OR)	phgPrP	0.5	Brun et al., 2007
Cow	bgTg008	bovine PrP (5 OR)	phgPrP	1	Brun et al., 2007
Cow	boTg007	bovine PrP (7 OR)	phgPrP	4	Castilla et al., 2004b
Cow	boTg006	bovine PrP (7 OR)	phgPrP	6	Castilla et al., 2004b
Cow	boTg012	bovine PrP (10 OR)	phgPrP	1	Castilla et al., 2005
Mink	Mk7	mink PrP	CosShaTet	2	Windl et al., 2005
Mink	Mk45	mink PrP	CosShaTet	1	Windl et al., 2005

Expressed PrP	Designation of transgenic line	Transgene	Transgenic Vector	Expression level	References
Cervidae	TgCerPrP1536	Cervid PrP	CosShaTet	5	Browning et al., 2004
Cervidae	TgCerPrP1534	Cervid PrP	CosShaTet	3	Browning et al., 2004
Cervidae	TgElk	ElkPrP	CosShaTet	2.5	LaFauci et al., 2006
Cervidae	TgElkPrP 12577	ElkPrP	CosShaTet	2	Tamguney et al., 2006
Cervidae	TgElkPrP 12580	ElkPrP	CosShaTet	2	Tamguney et al., 2006
Cervidae	TgElkPrP 3934	ElkPrP	CosShaTet	1.5	Tamguney et al., 2006
Cervidae	TgElkPrP 12584	ElkPrP	CosShaTet	3	Tamguney et al., 2006
Cervidae	TgDePrP 10945	DeerPrP (E226K)	CosShaTet	1	Tamguney et al., 2006
Cervidae	TgDePrP10969	Deer PrP (E226K)	CosShaTet	1	Tamguney et al., 2006
Cervidae	Tg Line 33	Deer PrP (G96)	phgPrP	1	Meade-White et al., 2007
Cervidae	Tg Line 60	Deer PrP (S96)	phgPrP	0.7	Meade-White et al., 2007
Cervidae	Tg Line 15	Deer PrP (G96)	phgPrP	0.5	Meade-White et al., 2007
Cervidae	Tg12	ElPrP-132M	phgPrP	2	Kong et al., 2005

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GLOSSARY

Prion	An acronym for “proteinaceous infectious particle.” All known mammalian prions contain misfolded isomers of a normal cellular protein (PrP ^C). Aggregates of the misfolded protein of sufficient quantity and size are associated with TSE infectivity and neurodegenerative diseases in both animals and humans. According to the methodology used for detection of the disease associated, misfolded protein, different terms have been used for its designation (See below). In mammals, prions are, at the present time, found primarily in nervous and lymphoreticular tissues. The preponderance of evidence, most recently reinforced by the creation of synthetic prions in different systems, suggests that prions may be the infectious agent of TSEs. However, there are some discrepancies between the presence of the protease-resistant form of the misfolded protein and infectivity, and a minority of respected TSE experts believe that the protein-only theory has not been proven beyond question (Erdtmann and Silvit, 2003).
PrP^{res}	Abnormally folded prion protein (PrP) that is partially resistant to proteinase K digestion and is strongly associated with TSE disease. It is sometimes not very faithfully used synonymously with PrP ^{Sc} .
PrP^{Sc}	Term originally derived from scrapie-associated PrP, but also more generally used in all TSEs. Abnormally folded prion protein that has a gradient of resistance to proteinase K digestion. It is associated with infectious potential and with TSE disease even in circumstances where it may be sensitive to proteinase K digestion.
PrP^d	Disease associated, abnormally folded prion protein. Sometimes this acronym is used when methods for detection of disease-associated PrP are employed that are not based on proteinase resistance nor infectivity assays, such as in immunohistochemistry.
PrP^{TSE}	TSE associated, abnormally folded prion protein. Sometimes “TSE” is replaced by the acronym of the respective disease, e.g. PrP ^{CJD} , PrP ^{GSS} , PrP ^{BSE} , PrP ^{Sc} , PrP ^{CWD} etc.
Transmissible Spongiform Encephalopathy (TSE)	A family of slowly progressive and ultimately fatal diseases of the central nervous system. They are characterized by transmissibility with a long incubation period, and spongiform degeneration of the central nervous system without inflammation and immunity response. Examples in humans include CJD and kuru. Among animals: scrapie and BSE. A synonym for TSE is prion disease.
Bovine Spongiform Encephalopathy (BSE)	A TSE of adult cattle. Contamination of MBM in feed with prions is considered to have caused the BSE epidemic that originated in the UK. Uncommon, presumably sporadic forms of BSE have been described more recently in TSE-infected cattle (Atypical BSE).
Scrapie	Designates a natural TSE of sheep and goats. This term covers a large variety of agents with different biological properties. Scrapie has been described in many parts of the world. It can be transmitted naturally or experimentally to other animals such as mice. It is the main prion source for experimental models of TSEs.
TSE agent	Causal agent of a TSE; see “prion” above.

Isolate	See definition in Section 2.2.1 of the Opinion.
Strain	See definition in Section 2.2.1 of the Opinion.
Cloned strain	See definition in Section 2.2.1 of the Opinion.

REFERENCES OF GLOSSARY

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