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Scientific Opinion on a request for a review of a scientific publication concerning the zoonotic potential of ovine scrapie prions

EFSA Panel on Biological Hazards (BIOHAZ Panel)

Abstract

The factors that modulate the transmissibility of Transmissible Spongiform Encephalopathies (TSE) and the approaches for the study of their zoonotic potential are reviewed. The paper '*Evidence for zoonotic potential of ovine scrapie prions*' by Cassard et al. (2014) is scientifically appraised, focussing on the experimental design, the results and the conclusions. The paper provides evidence in a laboratory experiment that some Classical scrapie isolates can propagate in humanised transgenic mice and produce prions that on second passage are similar to those causing one form of sporadic Creutzfeldt-Jakob disease (sCJD). It is concluded that the results from the study raise the possibility that scrapie prions have the potential to be zoonotic, but do not provide evidence that transmission can or does take place under field conditions. The conclusions of the 2011 ECDC-EFSA '*Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans*' are reviewed in the light of the new scientific evidence available since its publication. This supports and strengthens the conclusions of that opinion with regard to the potential for some animal TSE to be zoonotic, but does not provide evidence of a causal link between Classical or Atypical scrapie and human TSE. Current evidence does not establish this link, and no consistent risk factors have been identified for sCJD. The possibility of scrapie-related public health risks from the consumption of ovine products cannot be assessed. Recommendations are formulated on further studies and data that are needed to investigate the zoonotic potential of animal TSE and to estimate the amount of infectivity from TSE-infected products sourced from small ruminants and entering the food chain in the European Union.

Keywords: Atypical scrapie, Classical scrapie, Creutzfeldt-Jakob disease, transmissible spongiform encephalopathy, zoonosis

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Correspondence: biohaz@efsa.europa.eu

Panel members: Ana Allende, Declan Bolton, Marianne Chemaly, Robert Davies, Pablo Salvador Fernandez Escamez, Rosina Girones, Lieve Herman, Kostas Koutsoumanis, Roland Lindqvist, Birgit Nørrung, Antonia Ricci, Lucy Robertson, Giuseppe Ru, Moez Sanaa, Marion Simmons, Panagiotis Skandamis, Emma Snary, Niko Speybroeck, Benno Ter Kuile, John Threlfall, and Helene Wahlström.

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Summary

Following a request from the European Commission (EC), the EFSA Panel on Biological Hazards (BIOHAZ Panel) was asked to deliver a scientific opinion on the review of a scientific publication concerning the zoonotic potential of ovine scrapie prions.

The EC asked the BIOHAZ Panel to scientifically appraise the paper '*Evidence for zoonotic potential of ovine scrapie prions*' by Cassard et al. (2014), considering the limitations, assumptions and uncertainties associated with the study design and outputs. In addition, the BIOHAZ Panel was asked to advise whether the outcomes of the 2011 EFSA-ECDC '*Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans*' (EFSA BIOHAZ Panel, 2011), with regard to the zoonotic potential of both Classical and Atypical scrapie, were still valid. Finally, the BIOHAZ Panel was requested, based on the answers to the above questions, to advise whether the natural exposure of consumers to ovine products represents a non-negligible risk for public health.

This Opinion reviews the factors that modulate the transmissibility of animal Transmissible Spongiform Encephalopathies (TSE) and the approaches for the study of the zoonotic potential of TSE. It is concluded that there is no evidence of an absolute species barrier. Many factors influence the ability of any TSE agent to infect a host, regardless of whether the infection occurs across a species barrier, and it is impossible to define an experimental model that encompasses this potential variability and to directly measure zoonotic potential.

The scientific literature available since the publication of the 2011 EFSA-ECDC Joint Scientific Opinion is also reviewed to allow the appraisal of the paper by Cassard et al. (2014) as well as the review of the conclusions of that Scientific Opinion in the context of current knowledge.

The publication by Cassard et al. (2014) is reviewed, focussing on the experimental design, the results and the conclusions. The paper uses a combination of intracerebral inoculation, transgenic mice overexpressing human prion protein and serial passages that maximises the chance of detecting the propagation of TSE agents, but does not mimic natural exposure. It provides evidence in a laboratory experiment that some Classical scrapie isolates can propagate in humanised transgenic mice and produce prions that on second passage are similar to those causing one form of sporadic CJD (sCJD). This Opinion concludes that the paper under appraisal raises the possibility that scrapie prions have the potential to be zoonotic, but does not provide evidence that transmission can or does take place under field conditions.

The conclusions of the 2011 ECDC-EFSA Joint Scientific Opinion are reviewed in detail. Most of the conclusions formulated in that Opinion remain valid at present; only four require minor amendments. The new scientific evidence available supports and strengthens the conclusions of the previous Opinion with regard to the potential for some animal TSE to be zoonotic, but does not provide evidence of a causal link between Classical or Atypical scrapie and human TSE.

When considering the public health risks associated with exposure of consumers to scrapie agents through ovine products, the BIOHAZ Panel indicates that the level of exposure is largely determined by the prevalence of the disease in ovines and by the amount of infectivity in ovine tissues entering the food chain. The latter is reduced by the current specific risk material (SRM) measures. From the available epidemiological evidence it is not possible to conclude that the exposure of consumers to ovine products has resulted in the transmission of prion diseases to humans. A quantitative assessment of the overall amount of infectivity from TSE-infected ovine products entering the food chain would require data on infectivity distribution in small ruminants, disease frequency at population level, and sensitivity of detection of TSE-infected small ruminants at slaughter, among others. Data on these parameters and a mathematical model developed and applied previously by EFSA could be used for this purpose. An individual exposure to scrapie agents could be assessed only by combining the results from such an assessment with additional information such as consumption data and ovine product information.

It is concluded that current evidence does not establish a causal link between scrapie and sCJD, and that the possibility of scrapie-related public health risks from the consumption of ovine products cannot be assessed.

Recommendations are formulated on further studies and data that are needed to investigate the zoonotic potential of animal TSE and to estimate the amount of infectivity from TSE-infected products sourced from small ruminants and entering the food chain in the European Union.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

On 16 December 2014, an article titled *'Evidence for zoonotic potential of ovine scrapie prions'* was published in the scientific journal Nature Communications. This article provides the result of intracerebral inoculations of humanised transgenic mice (tgHu) with: (i) distinct ovine scrapie isolates, (ii) human prions, and (iii) cattle BSE prions. According to the authors, the study showed that the serial transmission (two passages were necessary) of different scrapie isolates in humanised transgenic mice led to the propagation of prions that were phenotypically identical to those that cause sporadic Creutzfeldt Jakob Disease (sCJD) in humans.

In 2011, EFSA and ECDC's joint scientific opinion on any possible epidemiological or molecular association between TSE in animals and humans[1] concluded that there is no epidemiological evidence to suggest that Classical scrapie is zoonotic, and that transmission data for evaluating the zoonotic potential of Classical scrapie in primates and tgHu were extremely limited or not yet available. It also concluded that the only TSE agent demonstrated to be zoonotic is the Classical BSE (C-BSE) agent.

EFSA is asked, in accordance with Article 29 of Regulation (EC) No 178/2002, for a scientific opinion addressing the following elements:

1. to scientifically appraise the publication, considering the limitations, assumptions and uncertainties (e.g. as regards how well the protocol of the study represents the human species barrier and as regards how well the experimental inoculation route employed represents exposure under natural conditions) associated to the study design and the outputs;
2. advise whether the outcome of the 2011 EFSA-ECDC joint opinion with regards to the zoonotic potential of scrapie is still valid in the light of the outputs of this study;
3. based on the answers to Terms of Reference 1 and 2, advise whether the natural exposure of consumers to ovine products represents a non-negligible risk for public health.

1.2. Interpretation of the Terms of Reference

Term of reference (ToR) 1 focusses on the appraisal of the new evidence presented in the paper by Cassard et al. (2014), which deals with Classical scrapie only. After discussion with the requestor of the mandate (European Commission), it was agreed to include Atypical scrapie in the review of the outcome of the 2011 Scientific Opinion (ToR 2), and therefore to expand the remit of the mandate to the investigation of the zoonotic potential of both Classical and Atypical scrapie.

1.3. General considerations on the study of TSE and their transmissibility

1.3.1. The species barrier

There are many factors which influence the ability of any TSE agent to infect an animal, regardless whether the infection occurs across a species barrier or not, such as the amount of agent present, the age of the animal challenged, and the possible potentiating effects of intercurrent disease or injury. These and many other factors have been speculated to affect the success of infection following exposure, but the precise roles and interdependence (if any) of these factors are not clear.

Infectivity is a measure of the ability of a disease agent to establish itself in a host. In the case of prions, infectivity may be estimated and expressed as ID_{50}, but it has been also shown that infection is possible at the very low dose of a 1 000-fold dilution of the ID_{50} (Fryer and McLean, 2011). The observed ID_{50} seen in one host or experimental animal model cannot be extrapolated to other agent/host combinations, and in particular, cannot be used to estimate the level of infectivity for

[1] EFSA Panel on Biological Hazards (BIOHAZ); Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans. EFSA Journal 2011;9(1):1945. [111 pp.] doi:10.2903/j.efsa.2011.1945. Available online: www.efsa.europa.eu/efsajournal

humans. It cannot either be extrapolated from one isolate to another. When referring specifically to the level of infectivity of TSE agents in the food chain, this is usually expressed as an estimated ID_{50} in the context of the experimental model used (e.g. bovine ID_{50} ($BoID_{50}$), or humanised transgenic mice ID_{50} ($tgHuID_{50}$)).

The infecting abnormal prion protein (PrP^{Sc}) must be able to 'convert' the cellular prion protein (PrP^{C}) to PrP^{Sc}, at a rate which enables accumulation of sufficient PrP^{Sc} to cause disease within the life-span of the host.

The host factor that has been shown to play a very key role in the overall susceptibility to TSE is the amino acid sequence of the host prion protein (PrP), coded for by the *PRNP* gene, and its associated species-specific polymorphisms. Early studies suggested that the cross-species barrier resides essentially in the differences of the PrP primary structure between the host and donor species (EFSA BIOHAZ Panel, 2011). Even single amino acid divergences may therefore have a major impact on transmission efficiency.

Host genetics substantially influence these diseases: in humans, familial prion diseases are closely associated with mutations in the *PRNP* gene, and the methionine/valine polymorphism at codon 129 appears to influence susceptibility, incubation period and in some respects disease phenotype. Several human forms of TSEs, such as Gerstmann-Sträussler-Scheinker (GSS) syndrome and fatal familial insomnia (FFI), have been shown to be genetic disorders relating to point mutations of the *PRNP* gene (Kovacs et al., 2002, 2005). *PRNP* gene variation in animals can be used to breed for resistance as a key disease control strategy in small ruminants (EFSA BIOHAZ Panel, 2014).

How or indeed whether it is the accumulation of PrP^{Sc}, the loss of function of PrP^{c} or some other factor associated with disease what results in the development of clinical disease, is not known. However, the presence of PrP^{c} is critical for successful infection. Animals which do not express PrP cannot be infected, but are nevertheless relatively normal (Benestad et al., 2012).

For abnormal PrP to reach the nervous system and for disease to develop, many events have to occur (Figure 1):

- There must be exposure to a sufficient dose of the agent. There is no data, for human TSE, on the dose-response relationship, or whether this might vary for different agent strains. It is also unknown if different doses would be required for an effective infection to occur depending on the different host variables listed below. For instance a higher dose might be required to effectively infect an older person, and vice versa. At present, the potential exposure of humans is minimized by the controls in place to reduce disease prevalence, namely feed bans, breeding for resistance and culling, and by removing from the food chain the tissues most likely to carry the greatest infectivity in any animals slaughtered for human consumption (Specified Risk Material (SRM) regulations).
- The agent must be taken up from the gastrointestinal tract. Although there is a body of work on oral exposure to scrapie and Bovine Spongiform Encephalopathy (BSE) agents (Head and Ironside, 2007), the point of entry through the gut wall is as yet not defined. It is speculated that the PrP^{Sc} and scrapie infective material may exploit the physiological process of macromolecular uptake across the gut (Akesson et al., 2012). M cells in particular have been demonstrated to modulate PrP^{Sc} uptake (Donaldson et al., 2012), and there has recently been some preliminary protective effect demonstrated from mucosal immunisation for Chronic Wasting Disease (CWD) (Goni et al., 2015). The expression of PrP^{c} in the gut and in the enteric nervous system (ENS) is essential for cell-to-cell transmission (Natale et al., 2011) and successful infection. Genotype therefore would also have a role to play at this point. All of these data have been generated in experimental challenges of ruminants or mice, so care must be taken when extrapolating to human gut.
- For sheep, cattle and humans alike there is a modelled association between the development of the gut-associated lymphoid tissues of Peyer's Patches (PP) and susceptibility to natural TSE infection. This association may explain the observed changes in susceptibility with host age (St. Rose et al., 2006).
- It has also been speculated that entry might be facilitated by other 'co-factors' such as mechanical loss of mucosal integrity within the gastrointestinal tract, and/or co-infection with

some unrelated pathogen, but no conclusive evidence has ever been found to support any specific contributing factors. However, gastrointestinal nematodes have been shown to modify host susceptibility to scrapie (Gruner et al., 2004). Differences in normal host physiology may also influence the outcome of exposure.

- The agent must enter the nervous system, and be successfully transported to the neuronal cell bodies in the central nervous system (CNS).

All of these stages are multifactorial.

The presence/absence of a species barrier does not in itself control the success of infection, or the resulting incubation period. For example, BSE caused variant Creutzfeldt-Jakob disease (vCJD) through oral transmission across a species barrier, with a likely wide exposure of the population, but only a few human cases have been identified, and in general a young age at onset (i.e. with an incubation period substantially shorter than average lifespan). By contrast, another TSE, kuru, is an example of transmission through ingestion of infectious material without a species barrier (human to human), and with a recorded incubation period of 5–50 years (Liberski, 2013).

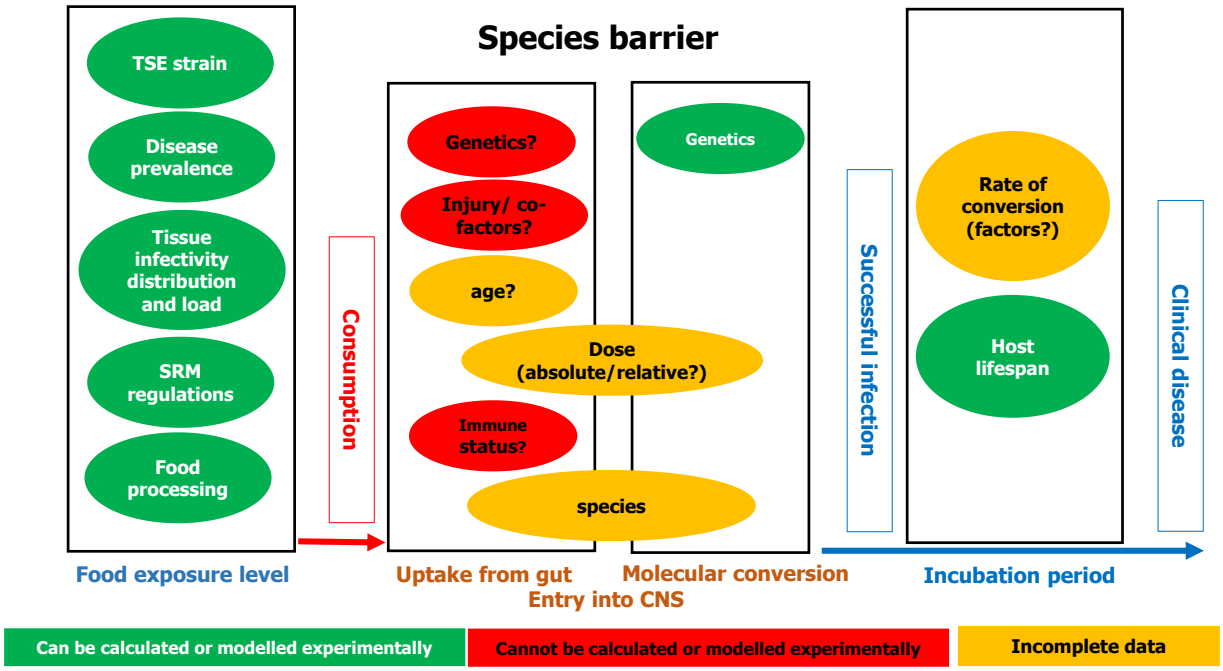

Figure 1: Schematic of the various factors which modulate the zoonotic potential of an animal TSE

It is therefore impossible to define an experimental model that encompasses all this potential variability and that directly measures the likely transmission across a species barrier (i.e. all the components required to achieve successful infection, see Figure 1). Trying to assess transmissibility to humans, i.e. zoonotic potential, directly, is even more difficult.

Many *in vivo* experimental models have been developed to investigate the interaction of TSE agents with human PrP (see Section 1.4.1), in particular transgenic mouse models. Mouse physiology and longevity are very different to that of humans, and are deemed by some to be an inadequate model for the direct study of TSE transmissibility. For this reason non-human primates, which have a longer life-span and substantial homology with man, have also been used in the study of these diseases, but such studies are necessarily and severely limited by considerations of both ethical and financial costs of such approach.

1.3.2. Agent strains

The effectiveness of the species barrier is determined not only by the host *PRNP* gene but also by the infecting strain of TSE agent.

A 'strain' is currently defined by its phenotypic characteristics in the natural host, which include the presenting clinical signs, histopathological lesion profile and immunopathology, the biochemistry of the PrP^{Sc} (e.g. protease sensitivity, molecular migration on Western blot, glycosylation patterns), and the biological characteristics of the agent in bioassay (i.e. relative incubation period, absolute susceptibility of experimental hosts), as well as the experimental host phenotype (e.g. lesion profile, immunopathology and PrP biochemistry).

Not all these parameters are necessarily stable for each agent. On occasions, isolates may change some or all of these phenotypic characteristics following either intra- or inter-species transmission (Simmons et al., 2015).

Agent strains in sheep

Based on strain typing studies in mice it is well documented that more than one TSE strain is responsible for Classical scrapie in sheep (Bruce et al., 2002; Bruce, 2003; Thackray et al., 2011, 2012; Beck et al., 2012a; Cassard et al., 2014). However, the exact number of Classical scrapie strains is unknown despite characterisation of several scrapie isolates in both wild type and transgenic mouse models. In addition, any direct correlation between Classical scrapie strains that have been characterised in wild type mice and those that have been identified in transgenic mice is undetermined. Therefore strains identified in wild type and transgenic mice have to be considered separately.

Historically, over 20 distinct mouse adapted laboratory Classical scrapie strains have been described in wild type mice (Bruce, 2003). These strains were isolated originally from less than 20 source animals, sheep or goats, and have had a complicated passage history in rodents (Bruce, 2002). Therefore they retain little resemblance to the properties of the agent in the sheep, and cannot be considered to comprehensively represent the field situation. Since the BSE epidemic, further characterisation of naturally occurring Classical scrapie cases that were detected after 1985 was initiated to identify strain variation in natural Classical scrapie using both wild type and transgenic mouse models (Bruce, 2002; Bruce et al., 2002; Beck et al., 2012a; Thackray et al., 2011, 2012; Cassard et al., 2014). Remarkably, the studies conducted in wild type mice after 1985 revealed novel strains in addition to the 20 that were known historically, suggesting that there may be evolution of Classical scrapie strains (Bruce et al., 2002; Beck et al., 2012a).

One caveat of the traditional strain typing approach using wild type mice was that it relied heavily on the interpretation of incubation periods and lesion profiles from a combination of mouse lines. In parallel with the introduction of transgenic mice in strain typing studies, additional analytical methods were applied which increased the sensitivity and specificity of strain detection and characterisation. These methods include immunohistochemistry and/or histo- or PET-blot, and Western blot. Although these newer methods can also be applied to wild type mice, they are not part of the official strain typing approach so the strains identified historically remained defined by their relative incubation periods and lesion profiles. However, successful attempts have been made to apply some of these new techniques to wild type mice (Beck et al., 2010a, 2012a, b; Corda et al., 2012; van Keulen et al., 2014). With the introduction of transgenic mouse lines there has been no standardised designation of strains although at least two approaches have been suggested: one based on the immuno-histochemical characteristics of PrP^{Sc} in coronal sections of mice (Thackray et al., 2011, 2012), and the other based on a combination of incubation period and Western blot (Laferriere et al., 2013). However, most strains characterised in transgenic mice are designated according to the reference code of the ovine source from which they have been isolated. Despite this, in a limited number of studies, five different Classical scrapie strains have been reported in UK, six in France and one in Italy (Thackray et al., 2011, 2012; Pirisinu et al., 2013a; Cassard et al., 2014).

Thus, evidence derived from a limited number of classical isolates cannot be extended to represent the whole biological variability of Classical scrapie.

In contrast to Classical scrapie, no strain variability has been observed so far among isolates of Atypical scrapie (Le Dur et al., 2005; Griffiths et al., 2010).

Agent strains in humans

Several distinct forms of prion disease have been described in man. Familial Creutzfeldt-Jakob disease (fCJD), GSS and FFI represent the main phenotypes of genetic prion disease (Mastrianni, 2003). About half of the genetic cases identified by surveillance have a family history of the disease, but an unknown proportion of cases may also result from *de novo* gene mutation (Mastrianni et al., 2003). Inherited prion diseases are autosomal dominant mutations (point mutations P102L, D178N, E200K and 2-, 4- and 6-octapeptide repeat insertions). FFI and a genetic form of Creutzfeldt-Jakob disease (CJD178) are clinically different prion disorders linked to the D178N PrP mutation (Bouybayoune et al., 2015). The glycoform ratios of PrP^{Sc} associated with *PRNP* gene point mutations are distinct from those observed in other human TSE; patients with the same *PRNP* mutation can also propagate PrP^{Sc} with distinct conformations (Hill et al., 2006).

Creutzfeldt-Jakob disease, the most prevalent human prion disease, occurs globally in several forms, the most common one being sCJD, which represents around 90 % of human prion cases (Safar et al., 2015). Although the precise cause of this disorder is unknown, there is evidence of genetic predisposition to sCJD (Ironside, 2012). The genotype (M/M, M/V, or V/V) at polymorphic codon 129 of the human prion protein (PrP) gene and the type (1 or 2) of protease-resistant PrP (PrP^{Res}) in the brain are major determinants of the clinicopathological phenotypes of sCJD. According to this molecular typing system, sCJD has been classified into six subgroups (MM1, MM2, MV1, MV2, VV1, and VV2). Whether these PrP^{Sc} subtypes identified by proteolytic fragmentation represent distinct strains of sCJD prions has been debated, but recent data show that sCJD prions are characterized by structural differences that are responsible for distinct prion replication rates and unique phenotypic characteristics of the disease. For example, in patients homozygous for methionine in the *PRNP* gene, there are two major subtypes of sCJD: MM1 and MM2. These types differ with regard to the progression rate of the disease, pattern of proteinase K (PK)-resistant fragments of infectious prion protein aggregates PrP^{Sc}, neuropathological characteristics of brain lesions, and transmissibility properties in transgenic mice (Safar et al., 2015). Besides these pure subgroups, mixed cases presenting mixed neuropathological phenotypes and more than one PrP^{Res} type have been found in sCJD (Kobayashi et al., 2011). The six sCJD types differ among each other in several clinical and histopathological features, and some of them propagate in animal models as distinct prion strains (Moda et al., 2012). Although *PRNP* is the major genetic determinant to susceptibility in human prion diseases, there is evidence that polymorphisms in the regulatory region may influence the risk of sCJD (Sanchez-Juan et al., 2011; Bratosiewicz-Wasik et al., 2012) and a recent genome wide association study has linked the glutamate receptor pathway to sCJD risk (Sanchez-Juan et al., 2015). It is not clear whether studies in tgHu accurately model these non-coding region genetic determinants of sCJD risk.

Besides sCJD, a novel form of human sporadic prion disease designated variably protease-sensitive prionopathy (VPSPr) has been recently identified (Gambetti et al., 2011). When compared with sCJD, VPSPr displays 'atypical' histopathologic and clinical features and shows a quite distinctive Western blot profile of abnormal PrP comprising truncated fragments in the 8–17 molecular weight range that are not seen in classic CJD.

Acquired human prion diseases account for only 5 % of cases of human prion disease. They include kuru, iatrogenic CJD and a new variant form of CJD (vCJD).

Acquired cases have arisen from iatrogenic transmission via human-derived growth hormone, human dura mater grafts and, rarely, via corneal grafts or contaminated neurosurgical instruments (iCJD). As in sCJD, MM homozygosity at codon 129 of *PRNP* seems to predispose to the disease, although this is not absolute. Attempts to identify differences in clinical manifestations, neuropathological changes and pathological prion protein (PrP^{Sc}) between iCJD and sCJD have been unsuccessful (Xiao et al., 2014). However, while traditional molecular typing of PrP^{Sc} is incapable of distinguishing iCJD from sCJD, the use of fragment mapping and protein misfolding cyclic amplification (PMCA)-based assays can distinguish most cases of iCJD from sCJD (Xiao et al., 2014).

Kuru is a human TSE that was confined to the Fore people of Papua New Guinea. Infection with the kuru agent was linked to ritual cannibalism and probably spread into the bloodstream from superficial wounds smeared with infected brain and from direct consumption of infected tissues. The disease died out with the cessation of these rituals and hence is not created spontaneously by the human host. Wadsworth et al. (2008) demonstrated that kuru prions have prion strain properties equivalent

to those of classical (sporadic and iatrogenic) CJD prions and speculated that kuru may have originated from chance consumption of an individual with sCJD. However data from Manuelidis et al. (2009) indicate that the kuru agent is a unique geographic isolate unrelated to sCJD. It is also different from the vCJD agent, the geographic Japanese CJD agent, and representative scrapie agents derived from sheep and goats.

Finally, vCJD, an acquired form of the disease that originated from a bovine prion strain, also displays a clinical and pathological phenotype that is distinct from sCJD and other forms of human prion disease, with a young age at onset and a characteristic neuropathology, with florid plaques in the brain, widespread accumulation of PrP^{Sc} and thalamic gliosis (Ironside, 2012). This is also the only human TSE in which significant levels of PrP accumulation in lymphoid tissues is described. Western blot analysis of the brain shows a predominance of the diglycosylated form of PrP^{Sc}, in contrast to sCJD. All definite vCJD patients who have undergone genetic testing are methionine homozygotes at codon 129 in the *PRNP* gene, suggesting a susceptibility to vCJD in this genetic subset. However, recent retrospective studies of archived lymphoreticular tissue in the UK, where most vCJD cases have occurred, identified abnormal PrP accumulation in lymphoid tissue in a number of individuals, with no codon 129 bias – unlike the clinical cases (Gill et al., 2013). These cases indicate a possible prevalence of 1:2000, which is much higher than that of clinical disease. The clinical significance of PrP accumulation in human gut-associated lymphoid tissue (GALT) is not understood, but it may represent a consequence of widespread dietary exposure.

1.4. Approaches for the study of the zoonotic potential of TSE

Given the impossibility of performing TSE transmission studies in humans, there are two main approaches to assess the zoonotic potential of TSE. It is important to appreciate that these approaches attempt to answer two fundamentally different questions: the permeability of the species barrier, and the link between human and animal TSE cases. In the following two sub-Sections both approaches are discussed in more detail.

1.4.1. Modelling the permeability of the transmission barrier

The protein-only hypothesis postulates that the post-translational conformational conversion of the cellular prion protein (PrP^{c}) into its pathological isoform (PrP^{Sc}) is an autocatalytic reaction. In this model PrP^{Sc} itself is the infectious agent, acting as seed for the subsequent polymerization mechanism of PrP^{Sc} formation. In this context, the species barrier, which defines the susceptibility/resistance of a host against a certain prion infection, may depend on the compatibility between the PrP^{Sc} from one species and the PrP^{c} from another species (for review see Barria et al., 2012). It is now widely accepted that host susceptibility and agent strain variability are closely linked with the conformation of the prion protein. While the difference between normal and disease-related PrP is largely a shift from alpha helix to beta sheet conformations, there are additional variations that result in unique tertiary structures, which are believed to account for different prion strains. It is the success with which a particular disease isoform can interact and 'force' conformational change on the normal host protein which determines whether or not an individual will succumb to disease, and it is the resulting disease-specific isoform, created from the host protein, that determines the disease presentation. This is referred to as the 'molecular' species barrier, and it can be experimentally explored both *in vivo* and *in vitro*. The glycosylation of the PrP has also been shown experimentally to affect the ease with which interspecies transmissions can occur (Wiseman et al., 2015). The molecular permeability of the transmission barrier is an important factor which limits the propagation of prion agents among different species.

It is possible that a host PrP may only be able to adopt a limited number of isomeric variations, regardless of the conformation of the infecting agent. This may result in a phenomenon known as 'convergence', where field strains with distinct phenotypes in their original hosts present with a common phenotype when transmitted experimentally to a different single *PRNP* gene background (Beringue et al., 2007). However, we know from the variability of TSE presentation in man that human PrP is capable of producing several distinct PrP isoforms.

As described above, the single quantifiable parameter with a proven effect on susceptibility is host genotype, and consequently, many *in vivo* and *in vitro* studies have started the assessment of the zoonotic potential of animal TSE by looking as directly as possible at the effect of human PrP

genotypes on the amplification of PrP^{Sc} following direct contact with defined TSE isolates. In such studies, a failure to convert the human PrP^{c} into PrP^{Sc} could reasonably be assumed to indicate that any natural exposure of people to the agent in question would not result in disease, i.e. a demonstrated lack of zoonotic potential.

Successful amplification of PrP^{Sc} would indicate that the TSE strain has the potential to convert human PrP^{c}. However, a positive result in an amplification or transmission experiment only means that transmission can occur, not that it does occur in field conditions. There are a number of fundamental differences between natural exposure and experimental studies which should not be overlooked when comparing disease models with field cases. Extrapolation of any results back to the donor/host-species needs to be an objective process.

The experimental approaches available are transmission in humanized gene-targeted and transgenic mice, *in vitro* conversion of human PrP sequences, and primate models, all of them reviewed previously by the EFSA BIOHAZ Panel (2011).

Bioassay using genetically modified mice expressing human PrP is the best available experimental approach to investigate the molecular barrier *in vivo*. The development of transgenic mice has been of prospective value, but at the same time can be misleading. For example, gene-targeted transgenic mice with a single copy of the human PrP gene were not susceptible experimentally to BSE (Bishop et al., 2006) while at the same time, epidemiological and strain-typing studies were producing a very strong body of circumstantial evidence that vCJD was a consequence of BSE infection in man. Although PrP over-expression might circumvent the low susceptibility of gene-targeted tg mice, it is worth noting that an inevitable limitation of such transgenic mice is that only one human gene is present in the model, while disease susceptibility and incubation period are inevitably multi-factorial. Additionally, if the time taken for the conversion of human PrP^{c} to PrP^{Sc} exceeds the lifespan of the mouse, this may give a 'false negative' outcome.

On initial interspecies transmission of TSE, the incubation period is usually long, but on subsequent mouse-to-mouse transmissions (serial passages), the incubation period usually 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 serial passage, especially the host mouse PrP genotype, remain constant. In transgenic mice increased PrP expression can shorten further 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 multiple transgene copies.

The incubation periods in different species may be influenced by species-specific factors other than the strain of the prion agent or host PrP expression. For example, BSE has an average incubation period of 5 years in cattle, 10–15 years in humans, 1 year (first passage) or 3–5 months (after adaptation) in wild type mice. The same applies to other agents: scrapie (an Italian isolate) has an incubation period of 1.5 years after intra-cerebral challenge in sheep (Vaccari et al., 2007), but 200 days (first passage) or 100 days (second passage) in voles (Di Bari et al., 2008). The use of TSE transmission models might therefore be limited by the life span of the recipient host, which may be exceeded by the incubation period. Successful transmissions with very 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. However, the conclusion that transgenic mice overexpressing PrP^{C} lead to a considerable reduction of the incubation time is also a point still open to debate: indeed, while this is true for a number of mouse and hamster strains, this statement seems not to be true in all cases. As an example, the incubation time of BSE in tg mice which over-express mouse PrP (tga20) is similar if not longer than in wild type mice (Yokoyama et al., 2009).

In vivo methods using non-primates for the study of the molecular permeability of the transmission barrier

Transgenic humanised mice (tgHu)

One approach to look at the zoonotic potential of animal TSE experimentally is to investigate the molecular permeability of the transmission barrier by bioassay in transgenic mice expressing common human PrP variants, i.e. human PrP homozygotes (MM129, VV129) or heterozygotes (MV129). The main purpose of the generation of humanised lines has been to develop animal models that may reproduce the susceptibility of humans to various CJD strains. Such lines can also act as models to assess the ability of various animal prion strains to overcome the transmission barrier at the molecular level. As the M129 PrP allele is associated with susceptibility to BSE and vCJD, it has been the allele of choice for generating human transgenic mice. Transgenic mice expressing human M129 PrP have been shown to be able to propagate cattle BSE, the only animal TSE with confirmed zoonotic potential identified so far. In this context, BSE can be used as a benchmark against which to evaluate the zoonotic potential of other animal TSE agents.

The gene-targeted PrP humanised mouse lines ensure the same expression levels (x1) of the V129 and M129 PrP alleles. A genetic cross between these two lines generates heterozygotes that express the same levels of each allele and therefore they could represent a good model to study the properties of an MV129 substrate exposed to TSE. In overexpressing mouse lines the expression levels cannot be predetermined; they can only be assessed after the generation of the founder transgenic mice. Therefore, it is difficult to create heterozygote lines that can express both alleles at similar levels.

Bioassays in transgenic mice expressing human PrP are artificial systems either because of PrP overexpression or because of the intracerebral route of inoculation. As such, these systems cannot reproduce field conditions but merely measure the ability of TSE to propagate on human PrP sequences, which represent only a part of the overall zoonotic potential. For example, L-type Atypical BSE (L-BSE) was proposed to be more transmissible than C-BSE, due to the results obtained in tg650 mice (Beringue et al., 2008a). However, successful transmission of L-BSE to cattle as the natural host via the oral route has not yet resulted in clinical disease, even after very long incubation periods (Panelli et al., 2011). Thus, the results obtained by experimentation in genetically modified mice have to be interpreted with caution and cannot be directly translated into a quantitative assessment of the zoonotic potential of animal TSE.

In the last few years, a number of different papers have reported the results of bioassays in transgenic mice expressing human PrP challenged with animal TSE isolates, including Classical and Atypical scrapie. These results are briefly summarised in the sections below, with particular emphasis on small ruminant TSE. In order to facilitate the evaluation of data obtained by different transgenic mouse lines and to allow further comparisons with those reported by Cassard et al. (2014), the data summarised below are also reported in Appendix A.

Gene-targeted transgenic mice

Gene-targeted transgenic mouse lines expressing physiological levels of MM129, VV129 or MV129 human PrPs (HuMM, HuMV and HuVV) have been shown to be susceptible to infection with sporadic human prions, and to a lesser extent to vCJD, but were resistant to cattle BSE and CWD from cervids (Bishop et al., 2006, 2010; Moda et al., 2012; Wilson et al., 2012). Interestingly, despite their lack of susceptibility to cattle BSE, HuMM transgenic mice were partially susceptible to sheep-passaged and goat-passaged BSE prions (Plinston et al., 2011, 2014; Wilson et al., 2013).

In addition, these gene-targeted transgenic mouse lines were challenged with two natural sheep scrapie isolates (Plinston et al., 2011) and two natural goat scrapie isolates (Wilson et al., 2013). Limited information on the strain properties of sheep isolates was available. However, both isolates were from VRQ/VRQ sheep from the Neuropathogenesis Unit (NPU) flock (Edinburgh, UK), suggesting limited scrapie strain variability. One goat isolate was characterized by PrP^{Sc} biochemical properties with some resemblance to BSE. Overall, primary transmissions of scrapie isolates were unable to cause disease in any gene-targeted mouse line. The same mouse lines were also used to assess the potential of Atypical scrapie to cross the barrier to humans (Wilson et al., 2012). Following challenge with one of seven Atypical scrapie isolates, gene-targeted mice expressing human PrP did not show

any signs of disease pathology. Serial passages in the same transgenic mouse lines, which could allow the identification of subclinical infection, were not performed. Overall, these negative results show that a molecular barrier for replication of scrapie isolates in humans does exist. However, these results have to be interpreted cautiously in light of the low susceptibility of the gene-targeted mouse lines used, which have been previously shown to be resistant to intra-cerebral challenge with BSE too, despite its known zoonotic potential (Bishop et al., 2010). Thus, these findings *per se* cannot be taken as evidence of a zoonotic potential of classical or Atypical scrapie lower than that of BSE.

Overexpressing transgenic mice

Two mouse lines were developed at University College London (UK): tg35 over-expressing twofold human 129M PrP and tg152 over-expressing sixfold human 129V PrP. These transgenic mouse lines were susceptible to sCJD and vCJD human prions and to a lesser extent to cattle BSE, but were resistant to CWD (Hill et al., 1997; Asante et al., 2002; Wadsworth et al., 2004, 2008; Sandberg et al., 2010). Interestingly, compared to gene-targeted transgenic mice and to other overexpressing transgenic mice, in these transgenic mouse lines susceptibility to BSE was not increased after passage in sheep (Wadsworth et al., 2013).

Tg35 and tg152 mice have been challenged with five Classical scrapie and five Atypical scrapie isolates from sheep (Wadsworth et al., 2013). Although these isolates were not selected specifically because they represented different scrapie strains, the Classical scrapie isolates were from different geographical origins (UK or Germany) and from sheep with different PrP genotypes (ARQ/ARQ or VRQ/VRQ). Furthermore, limited information from previous experiments in ovinized transgenic mice suggests a certain degree of strain variability among the Classical scrapie isolates that were included in that study. All these sheep scrapie isolates produced no clinical prion disease or biochemical or histopathological evidence for subclinical prion infection in these overexpressing transgenic mice. Sub-passages in these mouse lines were not performed.

A transgenic mouse line, tg40, which expresses the human PrP M129 allele at the wild-type level in the mouse PrP-ablated background, was produced at Case Western Reserve University (CWRU) (Kong et al., 2005). These mice were susceptible to sCJD prions and partially susceptible to L-BSE, but not to CWD. Tg40 mice have not been challenged with small ruminant prions so far.

A transgenic mouse line that overexpresses human PrP M129 allele at a sixfold level on a PrP null background, tg650, was produced at the Institut National de la Recherche Agronomique (INRA) (Beringue et al., 2008b). These mice propagated human sCJD and vCJD prions without evidence of any transmission barrier (Beringue et al., 2008b), while they were much less susceptible to cattle BSE (Beringue et al., 2008a). Interestingly, tg650 mice were resistant to the H-type Atypical BSE (H-BSE), but fully susceptible to L-BSE, with no apparent transmission barrier (Beringue et al., 2008a). Furthermore, tg650 mice were more susceptible to sheep- and goat-passaged BSE than to cattle BSE (Padilla et al., 2011).

Another transgenic mouse line was produced at the Centro de Investigacion en Sanidad Animal (CISA) (Madrid, Spain). This mouse line expresses human PrP M129 approximately fourfold more than normal human brain tissue. tg340 mice were fully susceptible to sCJD and vCJD human prions, partially susceptible to goat-and-sheep-passaged BSE and showed very limited susceptibility to cattle BSE (Padilla et al., 2011; Cassard et al., 2014), while being fully resistant to H-BSE (Torres et al., 2014). Tg340 mice have been challenged with a single scrapie isolate from Ireland, which did not induce clinical or subclinical infection after primary transmission and second passage. In comparison, a cattle BSE isolate showed higher efficiency of transmission, with transmission rates of 1/6 and 4/4 after primary transmission and second passage, respectively (Torres et al., 2014).

Overall, these results in overexpressing transgenic mice could suggest that the molecular barrier for the propagation of Classical and Atypical scrapie in humans might be higher than that encountered by cattle BSE in individuals homozygous for M129. These results have to be interpreted with caution, in light of the limited number of mouse lines and Classical scrapie isolates tested. Furthermore, data from mouse lines expressing MV129 PrP are still lacking. A more detailed comparison of all the results obtained so far, including those reported by Cassard et al. (2014), is provided in Section 3.1 and Appendix A.

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). This can make experimental data very difficult to interpret. Similar phenotype shifts have also been reported occasionally in wild-type mouse models. For example, a natural scrapie isolate was reported to exhibit, after first passage the C57BL/6 mouse line, a phenotype similar to sporadic and iatrogenic CJD (Lasmezas et al., 2001).

In vivo methods using primates for the study of the molecular permeability of the transmission barrier

A small number of primate studies have been undertaken to try to approximate the human species barrier to some specific animal TSE challenges, but these studies are extraordinarily expensive, and take years to produce results. The degree of genetic homology between man and the other primates is variable, and in no case is there full homology (see EFSA BIOHAZ Panel, 2011 for details). Non-human primates share a high level of genome homology with humans (notably 96 to 99 % homology to the human sequence of PrP). The chimpanzee (*Pan troglodytes*) is the closest model to human, followed by old world monkeys such as macaques (*Macaca*). Squirrel monkeys *(Saimiri sciureus)* and mouse lemurs (*Microcebus*) are easier to house and handle but evolutionarily are farther apart from humans. This may influence the pertinence of those models for assessing zoonotic risks of animal TSE: 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., 2009). However, it is not the host PrP sequence alone that determines susceptibility, but a combination of agent strain and host as seen in other species. Given the similarities in terms of physiology and anatomy, primates are considered to be optimal models for assessing the risks of real transmission to humans in natural conditions. The lifespan of primates (especially old-world monkeys) may exceed several decades, allowing the expression of TSE with long incubation periods in those animals.

Historically, the first experimental evidence of the transmissibility of human TSE diseases, including sCJD (Gibbs et al., 1968; Gajdusek and Gibbs, 1972; Zlotnik et al., 1974, Brown et al., 1994; Goudsmit et al., 1980), were obtained in primate models. Clinical and pathological similarities between those experimentally induced diseases in primates and corresponding natural diseases in human on the other hand have been described.

C-BSE transmission has been demonstrated in different primate species, including marmosets (Baker et al., 1993), cynomolgus macaques (Lasmezas et al., 1996, 2005), lemurs (Bons et al., 1999) and squirrel monkeys (Williams et al., 2007). The secondary transmission of both macaque BSE and human vCJD to the same host, i.e. conventional mice, induced similar lesional profiles, supporting the hypothesis of a direct link between the BSE and vCJD agents (Lasmezas et al., 2001). However, even with the small number of animals challenged, there was some minor variation in outcome, which may be related to the fact that different source isolates were used. When cynomolgus monkeys homozygous for methionine at codon 129 were challenged orally or intracerebrally with bovine BSE (Yutzy et al., 2007; Holznagel et al., 2013) they developed spongiform encephalopathy without the florid plaques that characterise human vCJD (Ironside, 1998), whereas such plaques were described in a separate challenge of cynomolgus monkeys with BSE (Lasmezas et al., 1996). Western blotting demonstrated a PrP profile with characteristic non-glycosylated double bands in brain homogenates digested with PK. Unfortunately this study did not show comparative data for any human TSE in this model.

Foodborne BSE prions enter the CNS of macaques (*Macaca fascicularis*, cynomolgus monkey) via afferent neurons, spreading centrifugally to lymphoid tissue at an advanced stage of the incubation period. In asymptomatic animals PrP was detected in 50 % and 12 %, of gut- and tonsil-derived samples, respectively (Holznagel et al 2015). PrP was also detected in non-CNS tissues (spleen, lymph node, and marrow) in at least 50 % of these infected macaques (Holznagel et al., 2010). In clinical cases, prion load in the GALT system increased compared with preclinical cases and included PrP^{Sc} deposits in germinal centres of follicles in the GALT. PrP^{Sc} deposits in ganglia of the myenteric plexus were also detectable. Interestingly, animals which received multiple oral doses did not succumb to clinical disease, although PrP had been deposited in the GALT (Strom et al., 2014). This is in keeping with the observation of PrP in the lymphoreticular tissues of vCJD patients (Ironside, 2012), and may

indicate a possible explanation for the high prevalence of human appendix samples with PrP accumulation (Gill et al., 2013).

In a different study, the PrP^{Sc} profiles of C-BSE-challenged macaques were almost identical, and similar to the glycoforms in cattle brain although with higher ratios of the monoglycosylated fragment (Ono et al., 2011a). BSE and the sCJD control were very different in this study, but the sCJD was a human sCJD isolate and not a macaque-passaged one.

Montag et al. (2013) intracerebrally challenged six (MM129) macaques with classical bovine BSE. The resulting individual glycopattern and band migration of macaque-adapted BSE PrP was compared with that of human isolates of sporadic CJD (sCJD) type 1, sCJD type 2, and vCJD. PK-resistant PrP from BSE-infected macaques co-migrated with type 2 sCJD and was clearly distinguishable from type 1 sCJD. However, the glycosylation pattern of macaque-adapted BSE was comparable with vCJD.

L-BSE has been transmitted both intracerebrally and orally to macaques resulting in a spongiform encephalopathy which was phenotypically distinct (clinical, lesional and biochemical) from macaque BSE (Comoy et al., 2008; Comoy, 2010; Ono et al., 2011a, b). In one study (Ono et al., 2011b) the resulting Western blot profile was similar to that of the bovine donor, with only a slight upward shift in electrophoretic mobility towards that of C-BSE. No PrP was detected in the lymphoreticular system (LRS). Histology and biochemistry studies showed similarities between L-BSE-inoculated macaques and MM2 sCJD patients, with similar lesional profiles, and the same sensitivity to proteolysis (Comoy et al., 2008; Comoy, 2010). Moreover, a macaque inoculated with brain of a MM2 sCJD patient showed a lesion profile similar to L-BSE infected macaques (Comoy et al., 2009). Cattle-adapted transmissible mink encephalopathy (TME) also induced a rapid disease in cynomolgus macaques (Comoy et al., 2011a). The clinical features, lesion profile, and biochemical signature of the induced disease was similar to the features observed in animals exposed to L-BSE, suggesting a link between the two prion strains.

Results related to Classical scrapie transmission in primates remain very limited, and do not enable comprehensive evaluation of the transmissibility of Classical scrapie agents given their diversity. One study (Baker et al., 1993) reported a Classical scrapie isolate transmitting to two marmosets after intra-cerebral challenge. More recently, Comoy et al. (2015) describe the successful transmission of a Classical scrapie isolate (one of the panel of scrapie isolates described in Cassard et al., 2014) to a cynomolgus macaque after an extended incubation period (>10 years following intracerebral challenge). The pathology observed in this animal was unique in comparison to other animal prion diseases (C-BSE, L-BSE, TME) previously transmitted in this model. Again, unfortunately no direct comparison is made with human isolates.

Attempts to challenge primate models (MM129 cynomolgus macaques) with CWD by various inoculation routes are ongoing (Comoy et al, 2011b; Motzkus, 2011; Mussil et al., 2015). Transmission has not yet been successful and all animals are healthy at less than 4 years post-inoculation.

In vitro methods for the study of molecular permeability of the transmission barrier

Amplification *in vitro*

The exact processes behind the autocatalytic post-translational conformational conversion of PrP^{C} into PrP^{Sc} are still enigmatic. However, *in vitro* conversion assays, which mimic the model, can be used as tools to examine the effects of the species barriers more deeply.

The cell-free conversion assay was the first method to be established, but this assay is relatively inefficient. Further developments led to the development of the PMCA and more recently to the quaking-induced conversion (QuIC). The latter is aimed primarily at developing a specific and sensitive test for prion diseases. The summary below focusses mainly on the species barrier phenomenon examined *in vitro*. For a comprehensive overview on the background, developments and principles of the *in vitro* conversion assays please see the EFSA-ECDC opinion (EFSA BIOHAZ Panel, 2011).

Cell-free-conversion assay

Cell-free conversion assays have been used to show that the self-propagation of PrP^{Sc} may be the molecular basis of scrapie strains (Bessen et al., 1995). Studies which focused on the molecular details of the conversion itself have demonstrated the importance of additional intermolecular

interactions between PrP^{c} and PrP^{Sc} after the initial binding, as well as the necessity of the disulfide bond in this process (Herrmann and Caughey, 1998). Factors supporting (i.e. protein chaperones) or inhibiting (i.e. congo-red, porphyrine) the cell-free conversion have also been examined (Deb Burman et al., 1997; Demaimay et al., 1998; Caughey et al., 1998). Research addressing interspecies and intraspecies transmission barriers in the cell-free conversion assay has shown some resemblance to actual biological species barriers. Kocisko et al. (1995) have demonstrated that non-homologous conversion reactions were not successful. In addition, successful heterologous conversion reaction using different PrP^{Sc} seeds and recombinant PrP^{C} as a substrate seems to be less efficient as compared to intraspecies transmission (Raymond et al., 2000; Panza et al., 2010). Successful heterologous conversion reactions using murine PrP^{c} and recombinant PrP^{c} with different mouse scrapie strains and mouse BSE have shown that prion strain phenotypes are retained. As in field infections, intrinsic factors including polymorphisms in the host, and post-translational modifications of PrP^{c}, play a significant role in the transmissibility of prion diseases in cell-free conversion assays (Bossers et al. 1997, 2000; Priola and Lawson, 2001; Kupfer et al., 2007; Eiden et al., 2011). In particular the experiments working with different polymorphisms, separately or in combination, demonstrate a high concordance between *in vitro* and epidemiological data (Eiden et al., 2011). Cell-free conversion assays modelling human susceptibility to animal prion diseases are rare, but have demonstrated clear species barrier phenomena, with limited conversion efficiencies, by using strains other than BSE PrP^{Sc} as seed. (Raymond et al., 1997, 2000; Luers et al., 2013).

Protein Misfolding Cyclic Amplification (PMCA)

PMCA was developed in 2001 as a technique for the *in vitro* amplification of PrP^{Sc} by an unlimited continuing replication of the misfolded aggregates (Saborio et al. 2001). In particular, serial PMCA allows a distinct increase in the efficiency of amplification as compared to manual PMCA (Castilla et al. 2005a). The underlying mechanism of PMCA is analogous to DNA amplification by PCR and this strategy seems, at an accelerated rate, to mimic the fundamental steps of the conversion process that takes place *in vivo* (Saborio et al,. 2001; Soto et al., 2002). However, several authors have reported a *de novo* generation of infectious PrP^{Sc} in unseeded assays after many rounds of amplification. Moreover intracerebral inoculation of this *de novo* generated protein resulted in diseases with new disease phenotypes (Saa et al., 2006; Barria et al., 2009; Chianini et al., 2012; Wang et al., 2010, 2012).

The newly generated PrP^{Sc} produced in homologous conversion assays shares the same biochemical, biological and structural properties as the parental isolate (Castilla et al., 2008a; Green et al., 2008; Thorne et al., 2012; Vidal et al., 2013a; Levavasseur et al., 2014; Priem et al., 2014), although exceptions have been reported (Thorne et al., 2012). This reproduction of strain adaptation and molecular patterns indicate again that the strain determinants are largely encoded in the structure and folding of PrP^{Sc} (Castilla et al., 2008a).

As seen *in vivo* the strain and source of PrP^{Sc}, in particular the *PRNP* genotype of seed and substrate, has a clear influence on the amplification pattern obtained by PMCA (Bucalossi et al., 2011; Taema et al., 2012; Thorne et al., 2012; Krejciova et al., 2014; Priem et al., 2014). In this regard it is of interest that a switch of the conversion profile has been shown by the amplification of an ARR scrapie isolate in different sheep genotypes, which resulted in a conversion profile that resembled mostly that of ARQ scrapie (Bucalossi et al., 2011; Priem et al., 2014). The newly generated PrP^{Sc} was infectious for several species (Castilla et al., 2005b, 2008a; Green et al., 2008; Saa et al., 2012; Di Bari et al., 2013). Most of the strains used showed similar incubation periods after intracerebral inoculation with brain or PMCA material (for review, see Saa and Cervenakova, 2014). In most cases the amplification is more efficient when the molecular characteristics of the PrP^{Sc} seed and the PrP^{c} substrate match, but the PMCA is able to overrule the transmission barrier in several heterologous combinations of PrP^{c} substrates and PrP^{Sc} seeds, which resulted in an efficient amplification and sensitive detection of heterogeneous PrP^{Sc}. This effect has been shown with CWD in ferrets (Kurt et al., 2007) and prairie voles (Kurt et al., 2011), RML (a laboratory scrapie strain) in cervid transgenic mice (Green et al., 2008), mouse scrapie in hamster (Castilla et al., 2008b), and feline spongiform encephalopathy (FSE) in bovine transgenic mice (Eiden et al., 2010). Even PrP^{c} from dog and rabbit, species which are not susceptible to TSE under natural conditions, could be converted by using scrapie (rabbit, Chianini et al., 2012) or BSE prions as seed (rabbit and dog, Vidal et al., 2013b).

Striking results have been obtained by serial PMCA modelling interspecies transmission. Several authors have described an adaptation of the amplified PrP^{Sc} to the new host, a process which normally requires several subpassages in animals (Castilla et al., 2008b; Green et al., 2008; Meyerett et al., 2008; Barria et al., 2011; Kurt et al., 2011). In these experiments novel prion strains arose with distinct biochemical and biological properties, even with altered species barriers:

- Hamster PrP^{c} misfolded by mouse PrP^{Sc} generated unique prions, which adapted in successive rounds of PMCA (Castilla et al., 2008b).
- CWD prions in cervid transgenic mice showed an adaptation process with shortened incubation periods in the subsequent performed mouse bioassay (Meyerett et al., 2008).
- Serial PMCA amplification of CWD prions in vole brain substrate enhanced not only their infectivity for voles *in vivo*, but also showed a biochemical pattern different from voles infected with deer CWD (Kurt et al., 2011).
- An interesting switch phenomenon was seen in serial PMCA using cattle and ovine BSE as seed. In a VRQ substrate a switch to the biochemical and biological properties of scrapie occurred after five to six rounds of PMCA and the ability to convert human PrP^{c} was lost. A re-amplification of this PMCA product in a bovine PrP^{c} substrate was not possible, clearly reflecting the species barrier seen with scrapie in cattle. On the other hand, ovine BSE and cattle BSE prions, amplified in an ARQ substrate, retained their molecular characteristics. However this PMCA product contained an additional scrapie-like component (Krejciova et al., 2014).

A few studies have examined human prion diseases by PMCA. Jones et al. (2011) were able to amplify vCJD, sCJD and even some animal prion diseases by using human brain, platelets and brains from transgenic mice overexpressing human PrP as substrate. As described above, strain characteristics were retained. Similar to the results obtained for the sheep *PRNP* genotypes, the human polymorphisms play a crucial role in the efficiency of the amplification.

PMCA has been used to model human susceptibility to animal prion diseases. Using vCJD, BSE and ovine BSE as seed and the three major human *PRNP* polymorphic variants as substrate, the efficiency of amplification is similar to the preference of vCJD in these genotypes seen *in vivo*, with 129MM as the best, followed by 129MV and less efficient 129 VV (Jones et al., 2009).

Neither Classical scrapie, Atypical scrapie nor Atypical BSE (H- and L-type) were amplified in human brains and humanized mice (Jones et al., 2009; Barria et al., 2014a).

Using different human PrP overexpressing transgenic mice as substrate and vCJD, Classical and L-BSE as seed, the PMCA results reflect the *in vivo* transmission data known from the different mouse models (Levavasseur et al., 2014).

The results obtained with CWD are puzzling, clearly indicating that the strain and the stabilization of the strain play a major role in the interspecies transmission, in which the species barrier is a dynamic process that depends on the strain and moreover the degree of adaptation of the strain (Barria et al., 2011).

Initially, brain from humanised-transgenic mice of the 129M and 129V lines could not be seeded with CWD from white-tailed deer and mule deer (Kurt et al., 2009). In serial passages *in vitro* (as well as *in vivo*) using cervid-transgenic mice as substrate, CWD prions from a naturally-infected mule deer were capable of converting human PrP^{c} and the resulting strain showed distinct biochemical properties (Barria et al., 2011). Elk CWD converted human PrP^{c} from human brain, humanized mouse brain and human-derived PrP^{c} overexpressing cell lines in a *PRNP*-dependent manner, with higher efficiency for 129M. Most interestingly, the resulting PrP^{Sc} resembled that of sCJD of the MM1 subtype providing evidence that a switch of the phenotype had occurred (Barria et al., 2014b).

1.4.2. Establishing the link between human and animal TSE cases

The link between human and animal TSE cases can be addressed directly by conducting molecular/biological comparison to determine the biological and biochemical similarities of TSE isolates from two different species. It can also be addressed through epidemiological studies that could set hypotheses for putative risk factors via descriptive observational studies or via analytical studies aimed

at detecting significant associations between exposure (risk factors) and outcome (health event of interest).

Direct biochemical or biological comparison of animal and human TSEs

Biochemical comparison

The characterization of the biochemical features of PrP^{Sc} can be used to identify particular TSE agents. Indeed the biochemical form of prion protein deposited in the brain in vCJD patients was found to be indistinguishable from that in BSE (Collinge et al., 1996), and it appeared that the BSE agent had highly stable features even after passage in other, but not all, host species. This was instrumental for developing tests aimed at discriminating the BSE agent from other TSE agents, in particular in small ruminants (Gretzschel et al., 2005; Stack et al., 2009). However, this approach has important limitations, as the PrP^{Sc} signature associated with a particular TSE agent might also be influenced by the host PrP sequence and the tissue environment where the protein accumulates. Based on these uncertainties, the former EFSA-ECDC Opinion (EFSA BIOHAZ Panel, 2011) concluded that '*the 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*'.

Few studies have directly compared the biochemical features of PrP^{Sc} in small ruminants and human TSE isolates. One study analysed the conformational stability of human sCJD MM1 and MM2 and of sheep Classical scrapie (an Italian isolate), suggesting that these TSEs have different biochemical properties (Pirisinu et al., 2010). A subsequent study investigated the properties of PrP^{Sc} from Atypical scrapie in small ruminants as compared to atypical human prions, including GSS cases with different PrP mutations and VPSPr. This study analysed the protease-resistant core of PrP^{Sc} by epitope mapping and the conformational stability of PrP^{Sc} by a solubility assay (Pirisinu et al., 2013b). By these methods, it was found that the biochemical properties of PrP^{Sc} from Atypical scrapie and GSS with P102L mutation largely overlapped, but were distinct from the other human prions investigated. Direct biological comparison is needed to conclude that Atypical scrapie and some GSS cases might be caused by similar or identical TSE agents. Overall, these results *per se* are of little aid in identifying potential links between small ruminant and human TSE, when it must also be considered that GSS cases are caused by pathogenic mutations in human PrP and are thus considered to develop spontaneously.

Biological comparison

Comparison of the disease phenotypes resulting from inoculation of animal models with animal or human TSE isolates can identify potentially common TSE agents, which in turn can lead to suspicion of, or support for, a cause-effect relationship. For example, Bruce et al. (1997) compared BSE and vCJD in RIII mice, showing their biological identity, which was extremely useful for demonstrating that the same TSE agent was the origin of BSE in cattle and vCJD in humans.

In general, these studies are done by transmitting TSE agents from different hosts into a third species, usually laboratory rodents. However, the lack of susceptibility of conventional mouse models to most of the sCJD isolates (Bruce et al., 1997; Hill et al., 1997) and to several scrapie isolates (Bruce et al., 1997; Beck et al., 2010b), has limited the usefulness of this approach for investigating the potential similarities of small ruminant and human TSE agents.

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 were shown to propagate efficiently in this model (Nonno et al., 2006). Based on the limited number of scrapie isolates published so far (Di Bari et al., 2008; Pirisinu et al., 2013a), no convergence with previously published sCJD cases (MM1, MV1, MM2) and gCJD (E200K and V210I) was observed in voles (Nonno et al., 2006). This observation was also strengthened by the characterization of PrP^{Sc} after transmission in voles of Classical scrapie and sCJD (Pirisinu et al., 2010; Pirisinu et al., 2013a). Overall, the characterization of five scrapie isolates in voles has been published so far (from UK and Italy, with ARQ/ARQ, ARQ/VRQ or VRQ/VRQ PrP genotypes), and none of them showed convergence with sCJD MM1/MV1 or MM2. However, the limited diversity of human and animal TSE isolates tested so far does not allow definitive conclusions to be drawn at this stage.

Finally, if we consider that any zoonotic transmission is a first passage in humans of an animal TSE, a potentially powerful experiment to investigate if such zoonotic events occurred is to inoculate the suspect human material in the supposed species of origin (or tg mice expressing the relevant PrP) and assess the transmissibility - expected to be efficient - and the resulting disease phenotype - expected to be similar to known phenotypes from the supposed species of origin. For example, it has been shown that vCJD transmits efficiently in transgenic mice expressing bovine PrP (tgBov) and induces a disease indistinguishable from BSE, which has been taken as supporting evidence of an origin of vCJD from cattle BSE (Scott et al., 1999; Wadsworth et al., 2004; Bishop et al., 2006; Padilla et al., 2011).

This approach has recently been used to 'trace back' different experimental TSEs. For example, after passage in sheep, BSE was still able to transmit efficiently in tgBov mice, while scrapie encountered a significant transmission barrier in tgBov mice (Torres et al., 2014). The experimental transmission of L-BSE in sheep of different genotypes induced partially deviant phenotypes of disease in some recipient sheep; still, in all cases the infection was traced back to cattle L-BSE by bioassay in tgBov mice (Torres et al., 2014; Nicot et al., 2014).

Nicot et al. (2012) examined the possible relationship between lemur-passaged L-BSE and sCJD through comparative transmission characteristics in Syrian golden hamsters and tgOvPrP4 mice expressing ovine PrP (ARQ allele). In hamsters, transmission of the MM2-cortical sCJD agent was inefficient, whereas lemur-passaged L-BSE transmitted efficiently. While the transmission to mice was efficient even in the first passage, mean survival periods decreased on second passage inoculated with sCJD, unlike that of mice inoculated with L-BSE. Western blot analyses of PrP^{Res} from mouse brains showed partially similar biochemical features for MM2-cortical sCJD and L-BSE. These results suggest that L-BSE did not undergo major modifications after this cross-species transmission and indicates a clear biologic difference between MM2 cortical sCJD and L-BSE.

So far, this experimental approach has not been used to look for a potential small ruminant origin of human TSEs.

Epidemiological approach

There are numerous other indicators which have to be taken into account before a conclusion is reached regarding the zoonotic potential of scrapie, mainly analysis of the epidemiological evidence.

Epidemiology may provide different investigation strategies to address the possibility of a link between animal and human TSEs.

Analytical studies may identify associations between the occurrence of sCJD and exposure to scrapie. In ecological studies the unit of observation is the population. Disease rates (here the occurrence of sCJD) and exposure (here the occurrence of scrapie) are measured in each of a series of populations and their relation is examined. These studies can be done quickly and inexpensively by using readily-available information.

Based in part on the interspecies transmission observed in animal TSEs, Diringer (1996) hypothesised a link between familial CJD and scrapie, but a comparison of the number of sheep per human population scrapie prevalence and mortality rates for sporadic CJD, published in response, argued that the incidence of sporadic CJD was independent of exposure of the human population to sheep and scrapie (Will et al., 1996). The epidemiological situation has not changed and an update of the worldwide occurrence of the diseases (i.e. Classical and Atypical scrapie and sCJD) is provided below.

Analytical epidemiological studies, for example case-control studies, are carried out to test the association between potential risk factors and the occurrence of disease. A case-control study, shifting from the population to the individual level, compares exposure histories between disease cases and non-diseased controls. For instance case-control studies in the USA have demonstrated an increased sCJD risk by using a range of foodstuffs derived from species including cattle, poultry, pigs, oysters and clams (Davanipour et al., 1985, 2014), whereas case-control studies in Europe have not demonstrated a significantly increased risk of sCJD due to occupation in farming or consumption of sheep products and (Harries-Jones et al., 1988; Wientjens et al., 1996; van Duijn et al., 1998).

However there are a number of limitations to epidemiological investigations of TSE:

- *Reliability of disease occurrence estimates from surveillance data and inability to control for the effects of potential confounding factors.*

  It is essential to have valid estimates of the occurrence of both diseases. Bias can occur if disease ascertainment differs from one country to another. Moreover, allowance has to be made for the potential confounding effect of specific risk factors accounted for by appropriate standardisation, e.g. age distribution is a relevant factor for sCJD. On the other side, surveillance targeting in small ruminants may affect estimates of scrapie occurrence with the potential for misclassification (either differential or non-differential).

  With regards to scrapie, the sample-based design of active surveillance may affect the estimates of occurrence in terms of both validity, e.g. if national representativeness is not guaranteed, and precision, which depends on the sample size. Within the framework of the European Union (EU) active surveillance, standardisation of surveillance streams might allow some rough comparison of prevalence between countries. However other confounding factors must be taken into account, e.g. age at testing, within-flock testing sample size, genotype distribution, and may therefore affect such comparisons (EFSA BIOHAZ Panel, 2014). When dealing with global scrapie statistics there is an even higher level of uncertainty. The OIE[2] does not require active surveillance to be carried out, but relies on surveillance focused on the testing of animals displaying clinical signs compatible with scrapie (passive surveillance). That leads to the availability of sparse national data that may allow only for the detection, rather than the quantification, of the level of occurrence of the disease.

  With regards to sCJD, the main limitations of the available data come from the passive nature of the surveillance system established in the 1990s, with a heterogeneous potential for under-reporting among the participating countries. Moreover although age- and sex-specific or standardized incidence or mortality rates are available, the national summary estimates available for comparison are based on crude rather than standardised rates. When considering the situation of countries not involved in the European Creutzfeldt-Jakob Disease International Surveillance Network (formerly EUROCJD),[3] data are to be considered with caution as criteria and procedures applied at national level may differ, preventing any meaningful comparison.

  Risk factor analysis in sCJD largely depends on case-control studies, prone to bias, which have been recently reviewed. Caution in the interpretation of results has been suggested due to: methodological concerns related to recall, control selection, exposure assessment in life-time periods of different duration, out of time-at-risk of effect, or asymmetry in case/control data (de Pedro-Cuesta et al., 2012).

- *Scrapie diversity*

  Besides the main distinction between Atypical and Classical scrapie, the fact that Classical scrapie can be caused by different TSE 'strains' (see Section 1.3.2) makes meaningful interpretations of any epidemiological investigation more difficult. Currently the routine monitoring activities in the field do not allow the discrimination, identification and reporting of strains. Therefore no data are available to describe the geographical distribution of strains within sheep populations. Moreover, there is a potential for the co-existence and mixing of strains.

- *Hosts genetics*

  As mentioned, the actual distribution of sheep genotypes linked to scrapie susceptibility is not well known and may explain within-country differences in the distribution of the disease. Analogously, the geographical distribution of human carriers of the relevant genetic factors (*PRNP*-129 polymorphism) in relation to disease susceptibility to CJD within national populations is only known for a few countries (e.g. the UK).

[2] OIE Terrestrial Animal Health Code 2014, see http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_bse.htm
[3] http://www.eurocjd.ed.ac.uk

- *Lag time (potential incubation period of sCJD)*

  From an epidemiological point of view, the comparison of the epidemic curves of BSE and vCJD has provided additional support to the other evidence indicating an association between the two diseases. In the case of a zoonotic role for scrapie, the consequent incubation period of any associated form of sCJD is unknown, although by analogy to other human TSE (e.g. iatrogenic CJD, vCJD and Kuru, with incubation periods ranging from five to 30 years or more) the period in the past to focus on when looking at the potentially relevant occurrence of scrapie is uncertain. Moreover if a reasonable assumption indicates an order of magnitude of years, currently available and reliable time-series of scrapie occurrence do not cover the period before the enforcement of the EU active surveillance (2002).

- *Ecological fallacy (or aggregation bias)*

  Relationships observed for groups do not necessarily hold for individuals, and vice versa. According to Hennekens et al. (1987), the main limitation of ecological studies is their inability to link exposure to diseases in particular individuals. In other words, correlation data represent average exposure levels rather than individual values. It could be the case that individuals unexposed to scrapie were the ones who later died of sCJD, that is, the so-called ecological fallacy.

- *Exposure misclassification*

  Human exposure to infected tissues via the consumption of small ruminant meat products may not be directly associated with the prevalence of scrapie in the area where products are consumed, but may instead depend on how the products are distributed and marketed, and on the individual consumption habits.

The epidemiological distribution of Classical scrapie and Atypical scrapie

Classical scrapie

The worldwide national distribution of Classical scrapie is based on individual case reporting generally obtained through passive surveillance, rather than systematic studies based on effective monitoring. Australia and New Zealand state that they have been free of scrapie since detecting the disease in imported animals in the 1950s (Detwiler and Baylis, 2003). Over the last decades, through the websites of ProMED[4] and OIE,[5] the presence of Classical scrapie has been notified by a number of countries outside the EU, although the level of occurrence is not known: Brazil, Canada, China, Falkland Islands, Iceland, Israel, Japan, Northern Cyprus (Gurel et al., 2013), Palestinian National Authority (PNA), Russia, Switzerland, Tajikistan, USA.

A thorough description of the epidemiological situation of Classical and Atypical scrapie within the EU has been provided in a recent Opinion of the EFSA BIOHAZ Panel (2014): in particular it refers to the 2002-2012 period.

The geographical distribution of the disease was obtained from the results of active surveillance. This was enforced from 2002 (Regulation (EC) No 1248/2001[6]) as a sample-based, targeted monitoring i.e. based on rapid testing of a sample of apparently healthy animals slaughtered for human consumption and found dead on farm (fallen stock). Compared with data from passive surveillance based on mandatory reporting of clinical cases, active surveillance data have clearly demonstrated that earlier evaluations of the geographical distribution were largely underestimated or biased.

Over the period 2002-2012, Classical scrapie in sheep was reported in 17 Member States out of 27. Both the temporal trend and geographical distribution of Classical scrapie showed substantial heterogeneity across Member States. Among countries reporting a sufficient number of cases over the years, six Member States showed a statistically significant decreasing trend. Due to the different

[4] http://www.promedmail.org/
[5] http://www.oie.int/
[6] COMMISSION REGULATION (EC) No 1248/2001 of 22 June 2001 amending Annexes III, X and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance and testing of transmissible spongiform encephalopathies. OJ L 173, 27.6.2001, p. 12–22.

ascertainment rate of Classical scrapie in the two surveillance streams and the different distribution of the number of tests carried out in each stream by country or by year, a standardisation of prevalence is needed. Over the 11-year period the national stream-standardised prevalence rates were extremely heterogeneous. Over the same period, Classical scrapie in goats was reported in eight Member States and showed similar heterogeneity (EFSA BIOHAZ Panel, 2014).

Atypical scrapie

Atypical scrapie has been reported sporadically in a few non-EU countries: Australia, Canada, Falklands Islands (Epstein et al., 2005), Japan, New Zealand (Kittelberger et al., 2010). Within the EU-27, Atypical scrapie in sheep was reported in 21 Member States. Prevalence was quite homogeneous over stream, time and space, without any national epidemic (EFSA BIOHAZ Panel, 2014). The disease has never been reported in Cyprus, Latvia, Lithuania, Luxembourg, Malta or Romania and identified sporadically in Austria, Bulgaria, Czech Republic, Estonia and Slovenia. With regards to goats, Atypical scrapie was reported by five countries (Finland, France, Italy, Portugal and Spain), at a very low prevalence.

The epidemiology of sporadic Creutzfeldt-Jakob disease (sCJD)

Surveillance of CJD has been carried out in the majority of the EU Member States for many years and mortality rates for ssCJD are shown in Table 1.

Although there is year-to-year variation, the mean annual mortality rates are relatively consistent at 1–1.5 cases per million in the majority of countries, consistent with previous studies of sCJD in Europe (Ladogana et al., 2005). Lower rates in some Member States most likely reflect limited experience of CJD surveillance and/or restricted resources. Recent evidence suggests that mortality rates are determined by the intensity of surveillance, including the number of notifications, the number of *ante-mortem* tests for marker protein 14-3-3 and the proportion of suspect cases undergoing neuro-pathological examination (Klug et al., 2013a). Resources to carry out specialist investigations are not equivalent in all countries and *post-mortem* rates vary. In addition a firm classification as sCJD depends on *PRNP* analysis and cases of genetic human prion disease may be misclassified if genetic analysis is not carried out (Kovacs et al., 2005).

Despite these considerations, mortality rates are remarkably similar in the majority of countries in Europe (Kovacs et al., 2007; Gubbels et al., 2012; Jansen et al., 2012; Brandel et al., 2013) and are consistent with recent national surveillance data from Argentina (Begue et al., 2011), Australia (Klug et al., 2013b) and Japan (Nozaki et al., 2010). It is of note that Australia has never reported a case of Classical scrapie. Since 2011, case reports or case series on ssCJD have also been published from China (Gao et al., 2011), Chile (Cartier et al., 2012), Hawaii (Kojima et al., 2013), Korea (Lee et al., 2015), India (Biswas et al., 2013), Morocco (Hajjaj and Kissani, 2011), Pakistan (Ahmad et al., 2014), Peru (Torres-Ramirez et al., 2014), Mexico (Moreno et al., 2013), and Turkey (Atalay et al., 2013). This evidence is consistent with the observation that sCJD occurs worldwide and the consistency of mortality rates in systematic surveys suggest that, should there be an environmental risk factor for the development of disease, this must be ubiquitous and evenly distributed. Although the cause of disease cannot be established in any single case, the absence of any consistent risk factor in sCJD, including occupation and dietary exposures, together with the epidemiological data, is compatible with the hypothesis that the majority of cases of sporadic CJD arise as a spontaneous disease with no external source of infection.

Table 1: Reported sCJD annual mortality rates per million in EU Member States reporting data to the Creutzfeldt-Jakob Disease International Surveillance Network[(a)] (1999-2013)

Country	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	Mean Rate
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	0.75	2.13	2.88	1.88	1.41
Belgium	-	-	-	-	0.97	1.37	1.27	0.88	1.46	2.02	1.83	1.92	1.24	1.52	1.51	0.56	0.93	1.11	1.19	0.64	0.45	1.28
Cyprus	-	-	1.48	0	0	0	0	0	1.43	0	1.39	1.37	0	1.3	1.28	3.84	0	0	0	1.16	0	0.7
Czech Republic	-	-	-	-	-	-	-	0.19	0.78	0.59	0.78	1.08	0.98	0.97	1.07	1.05	1.43	1.23	1.68	1.14	1.52	1
Denmark	-	-	-	-	2.09	0.94	1.69	0.94	1.12	1.68	1.86	1.3	0.74	1.47	1.1	1.1	2.36	1.08	1.26	1.79	2.32	1.46
Estonia	-	-	-	-	-	-	-	-	-	-	-	0	0.74	0	0	0	0	0.75	0	0	0.75	0.22
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	1.31	1.68	1.07	2.2	1.45
France	0.59	0.76	1	1.14	1.34	1.35	1.53	1.45	1.79	1.74	1.75	1.57	1.31	1.96	2.17	1.65	1.77	2.34	1.7	1.61	1.21	1.51
Germany	0.44	0.85	1	0.93	1.3	1.4	1.25	1.36	1.6	1.3	1.1	1.5	1.4	1.5	1.5	1.5	1.1	1.3	1.33	1.1	n/a	1.24
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	n/a	n/a	n/a	0.62
Hungary	-	-	-	-	1.1	0.3	0.5	0.9	1.7	0.7	0.8	0.8	0.8	1.2	1.3	1.4	0.7	1.4	0.8	1.9	1.6	1.05
Ireland	-	-	-	0.55	0.55	1.62	0.27	0.79	1.3	1.28	0.5	1.73	1.21	0.94	0.71	1.18	0.47	0.24	1.31	1.31	1.53	0.97
Italy	0.47	0.58	0.49	0.89	0.83	1.11	1.28	1.04	1.51	1.32	1.37	1.33	1.85	1.62	1.64	1.54	1.78	1.88	2	1.8	1.5	1.33
Latvia	-	-	-	-	-	-	-	-	-	0	0	0	0	0	0.43	0.43	0	0.89	0.45	0.49	n/a	0.22
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	0.67	1.69	2.04	1.55	1.54	1.12
Poland	-	-	-	-	-	-	-	-	-	0.16	0.31	0.52	0.36	0.13	0.08	0.26	0.86	0.08	0.68	n/a	n/a	0.34
Portugal	-	-	-	-	0.5	0.6	0.5	0.5	1.6	0.6	1.05	0.57	0.95	0.48	0.76	0.38	0.57	0.19	1.23	1.14	n/a	0.73
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	0.92	0.55	1.1	0.55	0.73	0.67
Slovenia	0	0.5	1	0	0	0.5	1.5	0.5	2	2.5	1	1	1	3.5	1	1.5	1	0.5	0.5	3	2	1.23
Spain	0.54	0.43	0.51	0.69	0.79	1.57	1.27	1.14	1.57	1.36	1.44	1.36	1.81	1.53	1.76	1.37	1.4	1.28	1.26	1.06	1.08	1.2
Sweden	-	-	-	-	1.13	1.58	1.24	1.58	1.35	1.34	1.23	1.66	1.44	1.76	1.2	1.4	1.18	1.49	1.58	1.47	n/a	1.41
UK	0.62	0.93	0.6	0.69	1.01	1.09	1.06	0.85	0.98	1.23	1.33	0.84	1.11	1.12	1.05	1.4	1.29	1.35	1.42	1.46	1.59	1.1

n/a: not available; sCJD: sporadic Creutzfeldt-Jakob disease.

(a): Available online (accessed on 1 July 2015): http://www.eurocjd.ed.ac.uk/surveillance%20data%201.html

1.5. Concluding remarks

- There is no evidence of an absolute species barrier. There are many factors that influence the ability of any TSE agent to infect a host, regardless of whether the infection occurs across a species barrier.
- The host factor that has been shown to play a very key role in the overall susceptibility to TSE is the amino acid sequence of the host *PRNP* gene, and its associated species specific polymorphisms.
- For disease to develop there must be exposure to a sufficient dose of the agent, the agent must be taken up from the gastrointestinal tract, enter the nervous system and be successfully transported to the neuronal cell bodies in the central nervous system. The infecting agent must then be able to 'convert' the cellular PrP^{C} to PrP^{Sc} at a rate which enables accumulation of sufficient PrP^{Sc} to cause disease within the life-span of the host.
- It is impossible to define an experimental model that encompasses this potential variability and to directly measure zoonotic potential.
- The level of exposure to TSE agents influences the likelihood of successful infection. The level of exposure of consumers to TSE agents through oral exposure is largely determined by the prevalence of animal TSE and by the amount of infectivity in animal tissues entering the food chain. The latter is reduced by current SRM measures.
- A spectrum of strains is responsible for Classical scrapie in sheep, and there may be variability in properties that affect the ability to cross the species barrier. There is experimental evidence that some isolates may not be completely stable, and their fundamental properties may shift on transmission. There is also potential heterogeneity of geographical distribution of individual strains.
- Evidence derived from a limited number of classical isolates cannot be extrapolated to represent the whole biological variability of Classical scrapie. In contrast to Classical scrapie, no strain variability has been observed so far among isolates of Atypical scrapie.
- Several distinct forms of prion disease have been described in humans. Some are genetic in origin, and some are acquired. Creutzfeldt-Jakob disease, which represents around 90 % of human prion cases, occurs globally in several forms, sporadic CJD (sCJD) being the most common. sCJD has been classified into six subgroups that differ from each other and may propagate in animal models as distinct prion strains. Variant CJD (vCJD) is acquired from BSE, is distinct from sCJD and other forms of human prion disease, and is currently the only human TSE to be confirmed as zoonotic.
- The 'molecular permeability' of the transmission barrier, i.e. the conformational compatibility between the infecting prion strain and the PrP^{c} of the recipient species, is an important factor which limits the propagation of prion agents among different species. Successful amplification of PrP^{Sc} would indicate that the TSE strain has the potential to convert human PrP^{c}. However, a positive result in an amplification or transmission experiment only means that transmission can occur, not that it does occur in field conditions.
- The molecular permeability of the transmission barrier can be modelled experimentally by bioassay in transgenic mice expressing common human PrP variants. Transgenic mice expressing human M129 PrP have been shown to be able to propagate cattle BSE, the only animal TSE with confirmed zoonotic potential identified so far. In this context, BSE can be used as a benchmark to evaluate the zoonotic potential of other animal TSE agents.
- Based on a limited number of Classical scrapie and sCJD isolates investigated so far by transmission in voles, there is no evidence of any common strain involved in Classical scrapie and sCJD, but the number of isolates studied is far from comprehensive at this point.
- Both cell-free conversion assays and PMCA allow the examination of the molecular species barrier phenomenon *in vitro*. Serial PMCA is able to mimic the strain adaptation process seen *in vivo*, resulting in novel prion strains with distinct biological properties different from the original isolate. In this regard a switch of the phenotype has been described. BSE prions in

sheep VRQ substrate switched to scrapie and elk CWD prions in human substrate switched to sCJD.

- *In vitro* conversion of human PrP^c using Atypical and Classical Scrapie as substrate has not yet been described, but it has been shown for C-BSE, L-BSE and CWD. *In vivo* transmission of CWD to humanised mice has not yet been achieved, so the conversion outcomes are not exactly the same.
- The challenge of primate models with Classical BSE has consistently resulted in transmission of disease with features similar to vCJD. A small number of challenges with scrapie have also resulted in transmission, but the resulting disease does not resemble known human TSE. L-BSE has also transmitted, and showed some similarity to sCJD. Other animal TSE (e.g. CWD) are harder to transmit. These studies demonstrate that the human species barrier for animal TSE is not absolute, and that all can be demonstrated experimentally to have some zoonotic potential.
- Epidemiological investigations at population (ecological studies) or at individual level (case-control studies) have been carried out to investigate an association between the occurrence of sCJD and exposure to scrapie. From the available epidemiological evidence it is not possible to conclude that the exposure of consumers to ovine products has resulted in the transmission of prion diseases to humans, nor that Classical scrapie is zoonotic.
- A number of limitations to this epidemiological approach have been pointed out: the reliability of disease occurrence estimates from surveillance data, the diversity and possible geographical heterogeneity of scrapie agents, host genetics at a population level, the potentially variable lag time between the presence of scrapie in the food chain and the estimated hypothetical occurrence of any consequential zoonosis, and aggregation bias. The validity of case-control studies applied to sCJD may be influenced by recall and selection bias and by the inability to appropriately account for latency, age-related susceptibility and concomitant confounding factors.
- The global distribution of Classical scrapie mostly relies on data from passive surveillance: the occurrence of scrapie may be missed and limited quantification compromises inter-country comparisons. Within the EU, where active surveillance has been carried out for over 10 years, Classical scrapie shows a heterogeneous epidemiological distribution both in terms of presence of disease and of level of prevalence, whereas Atypical scrapie shows a relatively homogeneous prevalence in time and space.
- Surveillance of sCJD has been carried out in the majority of Member States for many years. Although there is variation from year to year, the mean overall annual mortality rates are relatively consistent at 1–1.5 cases per million, suggesting that should there be an environmental risk factor for the development of disease, this must be ubiquitous and evenly distributed. The absence of any consistent risk factor in sCJD, including occupation and dietary exposures, together with the epidemiological data, is compatible with the hypothesis that the majority of cases of sCJD arise as a spontaneous disease with no external source of infection.
- 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 (minimal exposure) as opposed to Iceland and Cyprus (high exposure) argue against this possibility.

2. Data and Methodologies

2.1. Data

The data used in Section 1.3 (General considerations on the study of TSE and their transmissibility) have been sourced via a literature search on the new evidence on the experimental studies showing the transmissibility of the TSE agents to humans, as described in Section 2.2. Additional data have been extracted from scientific papers that were out of the scope of the search due to year of publication (prior to 2010) or the subject (TSE pathogenesis).

The appraisal of the scientific paper of Cassard et al. (2014) has been conducted considering the data presented in it and the additional information provided by the corresponding author, consulted in the role of Hearing Expert by the Working Group drafting this Scientific Opinion.

2.2. Methodologies

The paper by Cassard et al. (2014) can only be assessed against the background of the state of knowledge about the transmissibility of TSE as a whole – the species barrier and the relationship between the strain of the agent and the host genotype. There is a full description of many of these parameters in the 2011 joint EFSA/ECDC opinion (EFSA BIOHAZ Panel, 2011), and the relevant key points and conclusions from that Opinion are re-iterated in this document for continuity and ease of reference. These conclusions are updated based on a review of the literature (2010–2015) in order to put in the context of current knowledge both the appraisal of the paper (ToR1) and the review of the 2011 opinion (ToR2)

A literature search was performed in the framework of this mandate to inform the review of the evidence in the scientific literature on the *in vivo* and *in vitro* experimental studies showing the transmissibility of the TSE agents to humans. In particular, the search was aimed at identifying new scientific evidence that has become available subsequent to the publication of the 2011 EFSA-ECDC joint scientific opinion, since these areas have been developed the most during recent years. The literature search was used to support the expert review of these areas, and additional scientific information known by the experts was also considered in the review.

The search string used for the literature search of *in vivo* transmission studies of TSE in animal models exploring the zoonotic potential of scrapie were: (BSE OR TSE OR scrapie OR CWD OR *CJD OR Nor98 OR Nor-98 OR spongiform encephalopa* OR 'chronic wasting disease' OR 'creutzfeldt-jakob' OR 'creutzfeldt jakob' OR prion OR prp*) AND (transmissible OR transmission OR transmitted OR transgenic OR barrier OR passage* OR tg OR humanised OR humanized). These terms were searched in the titles of the scientific publications. The search was conducted in the following databases: ISI Web of Knowledge: CAB Abstracts; Current Contents; FSTA; Journal Citation Report and Web of Science. The search was restricted to English language and from 1 January 2010, since previous studies were covered extensively by the EFSA BIOHAZ Panel (2011). A total of 529 references (1 April 2015) was retrieved and screened for studies of interest. A subset of 64 references were considered potentially relevant and reviewed.

The search string used for the literature search of *in vitro* transmission studies of TSE in animal models exploring the zoonotic potential of scrapie were: (BSE OR TSE OR scrapie OR CWD OR *CJD OR Nor98 OR Nor-98 OR spongiform encephalopa* OR 'chronic wasting disease' OR 'creutzfeldt-jakob' OR 'creutzfeldt jakob' OR prion OR prp*) AND (misfold* OR conversion OR 'in vitro' OR 'in-vitro' OR amplification OR passage* OR cycl* OR substrate OR *quic OR 'asa' OR pmca OR quaking). These terms were searched in the titles of the scientific publications. The search was conducted in the following databases: ISI Web of Knowledge: CAB Abstracts; Current Contents; FSTA; Journal Citation Report and Web of Science. The search was restricted to English language and from 1 January 2010, since previous studies were covered extensively by the EFSA BIOHAZ Panel (2011). A total of 503 references (26 March 2015) was retrieved and screened for studies of interest. A subset of 34 references were considered potentially relevant and reviewed in detail.

One search was performed to identify epidemiological studies exploring the association between scrapie and sCJD. The update on the section discussing epidemiological evidence has been conducted

based on the results of this literature search and on the expert opinion of the members of the EFSA Working Group on Zoonotic Potential Scrapie.

The search string used for the literature search on epidemiological studies on the association between scrapie and sCJD were: (scrapie OR risk*) AND (CJD OR sCJD 'Creutzfeldt Jakob disease' OR 'Creutzfeldt Jakob Disease' OR 'creutzfeldt jakob disease' OR 'Creutzfeldt-Jakob disease' OR 'Creutzfeldt-Jakob Disease' OR 'creutzfeldt-jakob disease'). These terms were searched in the titles of the scientific publications. The search was conducted in the following databases: ISI Web of Knowledge: CAB Abstracts; Current Contents; FSTA; Journal Citation Report and Web of Science. The search was restricted to English language and from 1 January 2010, since previous studies were covered extensively by the EFSA BIOHAZ Panel (2011). A total of 50 references (1 June 2015) was retrieved and screened for studies of interest. A subset of five references were considered potentially relevant and reviewed.

3. Assessment

3.1. Scientific appraisal of the publication by Cassard et al. (2014)

The paper by Cassard et al. (2014) studied the effect of the Met/Val codon 129 polymorphism on the susceptibility of tgHu mice by inoculating a panel of scrapie isolates, which represents a proportion of the diversity of Classical scrapie strains. The study also investigated the phenotypes of disease after serial passages of scrapie in tgHu mice in comparison to some sCJD types.

3.1.1. Appraisal of methods

Mouse lines used

The mouse lines used by Cassard et al. (2014), in particular tg650 and tg340, are well established and have been shown to be susceptible to different CJD and BSE strains. The tg650 mouse line overexpresses sixfold an M129 PrP allele; tg340 mice also overexpress fourfold an M129 human allele. A third mouse line, tg361, which overexpresses a V129 allele at similar levels fourfold as the tg340 line was also used. A breeding cross between these latter two lines provided mice that overexpress both M129 and V129 alleles at similar levels. The inclusion of the MV129 heterozygote mice represents an important point, as most previous studies have not modelled the transmission barrier in this PrP genotype. Although over-expression of PrP is not a natural condition in humans, and it might have impact on some biological parameters, this can be considered a scientifically appropriate approach to modelling the molecular barrier for transmission of scrapie in humans given the limitations of these transmission models as previously discussed (see Section 1.4.1).

Inocula used

The authors used different isolates of Classical scrapie, which were previously studied in other animal models and showed some degree of biological variability. The deliberate selection of biologically variable scrapie isolates represents an important new aspect compared to previous studies on the subject, given the known diversity within the group of TSE agents identified as 'Classical' scrapie (see Section 1.3.2). In similar previous studies, fewer isolates were used. No case selection will conclusively and comprehensively ever represent the total potential field exposure, but this study makes a good, rational and supported choice of isolates designed to be distinct from one another, to represent some of the possible range of field strains.

The inocula used were obtained from ARQ and VRQ homozygous sheep based on the biochemical characteristics of both the original sheep isolates, and the transmission patterns that they showed on primary isolation in different transgenic mouse lines.

Additionally, two different BSE isolates were included as positive controls to demonstrate the susceptibility of these tgHu mice to prion strains with known zoonotic potential. It is not explicitly stated that these are both C-BSE, although it is implied by the lack of classification. Given that they are the same 'strain', it would have been useful to know if there were differences for example in the

infectious titres which could have explained the differences in attack rates between the two controls that were seen in the tgMet129 mice.

Inoculation route

The method used (intracerebral inoculation of a 10 % brain homogenate) is widely used and accepted. In this particular experiment, the robustness of the results is enhanced as similar outcomes are observed in three different laboratories with two different transgenic lines, minimizing the possibility of any confounding effect or possible cross-contamination as a consequence of the inoculation procedures.

The isolates were inoculated by the intracerebral route whilst natural exposure in man involves the oral route. In this respect the inoculation route used does not represent an ideal strategy for the investigation of zoonotic potential since the involvement of the digestive system particularly of the GALT, the rest of the LRS, of ENS and peripheral nervous system have been bypassed by the direct deposition of the prions in the brain. Nevertheless, as the focus of the study was not to define the probability of transmission in field conditions but to model the molecular permeability of the transmission barrier, the intracerebral route is an accepted and appropriate choice.

Classification of TSE agents

The classification of TSE agents relies on the description of various phenotypic characteristics (as described in Section 1.3.2). In mouse bioassays, the incubation period, vacuolation lesion profile, immunopathology and PrP biochemistry are the most commonly presented data, and these are the characteristics that can be used to classify the infecting agents as similar to, or distinct from, one another. There is no universal agreement on how many or which parameters need to be included in such a comparison, hence different studies often use a different combination of approaches.

In this study, the transmission results are presented clearly and consistently. However, different sets of parameters are used at different points in the assay. For example, only incubation periods and Western blots were used to describe infected animals at primary challenge, largely because of the need to use tissue for subpassage. After second passage, the immunopathology of some passaged isolates is described, but it is not clear if this approach was applied to all mice. Again, characterization of the propagated strains relies mostly on incubation period and Western blot profile. However, comparison is made between the scrapie isolates and the vCJD and sCJD isolates in the same mice, but, with the exception of incubation period, which is the crudest of the phenotypic characteristics, data are not shown for the BSE isolate. There is no indication of which phenotype the inoculated BSE showed: vCJD and/or sCJD (as for example described by Asante et al. 2002). Data showing that BSE and vCJD could be classified as similar in this model and separate from sCJD, would have helped to counter any suggestion that the results might be explained by convergence. An additional analysis in depth of the single positive mice after first (MF17, PS21) and second passage (PS09, PS21, PS42) would have been interesting, but is missing, possibly because if only a single mouse was positive, it would have been used for further passages.

In summary, discriminatory testing, analysis of PK-resistance, epitope-mapping and glycoprofiling, as well as examinations for conformational stability, lesion profile and immunohistochemistry were, with exception of a single PET blot, not done (or at least not described). This PET blot, on the other hand, is from bioassays performed with material from serial passages.

Species barrier

Mouse bioassays are very artificial systems and results obtained by (serial) passages should be interpreted carefully. In particular, there is some divergence of opinion about the interpretation of sub-passages as a proxy for longevity, rather than just a means of identifying sub-clinical infection in the donor animal. Little is known about the dynamics of PrP conversion in the presence/absence of PrP^{Sc}, so it may be that in an individual long-lifespan host there may be additional rate-limiting factors at a cellular level, which are unknown or not understood. Cassard et al. (2014) used overexpressing transgenic mice, and the results largely depend on the outcome of a second or third passage (see Table 2). Additionally, the second passage in this study was performed with pooled brains from mice, which survived the first passage for longer than 500 days. However, there is some inconsistency in the different serial passages applied, as not all inocula were sub-passaged (see Table 2), and some

sub-passages were not made using pools. This apparently reflects the different practices in the different contributing laboratories, and while it might affect the infectious titre, it should not influence the biological properties of the isolate.

The use of a 'blind' second passage (i.e. sub-passage in the same mouse line of brain homogenates from negative mice deriving from the primary transmission) provides robust data on the level (if any) of subclinical disease after primary transmission, and is a good scientific addition to this body of work. However, it is not explained why blind sub-passages were not performed on the negative BSE control.

3.1.2. Appraisal of results and conclusions of the publication

The mouse lines used in the study expressed human PrP approximately fourfold more than is seen in normal human brain tissue. Transgenic mice with M129 PrP have been previously shown to be fully susceptible to human TSE, such as sCJD and vCJD and, to a much lesser extent, cattle BSE. This study provides evidence that tgMet/Val129 are susceptible to sCJD prions, partially susceptible to vCJD prions but resistant to cattle BSE. Transgenic mice with V129 PrP were susceptible to sCJD prions, but not to vCJD or BSE.

When these mice were challenged with six Classical scrapie isolates indicative of different scrapie strains, none induced clinical disease in any of the recipient mouse lines. However, a single scrapie isolate produced sub-clinical infection in two out of six tgMet/Val129 mice. Furthermore, blind second passages suggested that low levels of sub-clinical infection were present in tgMet129/tg340 inoculated with three further scrapie isolates. The efficiency of transmission in tgMet129/tg340 can be compared with that of two cattle BSE isolates, which gave no transmission in one case, and low transmission rate (1/6) in the other. Blind second passages were more efficient in BSE than in scrapie, with overall rates of transmission of 9/12 mice in BSE and 4/24 mice in scrapie (see Tables 4–6 in Appendix A). It is not clear why such sub-passages were not carried out for the BSE isolate in the Met/Val129 mice.

Another transgenic mouse line overexpressing human M129PrP allele at a sixfold level, tg650 mice, was challenged with four Classical scrapie isolates (three in common with the first group of challenges, and one further isolate (O100) for which no source phenotypic information is given. Two isolates induced sub-clinical disease at a low rate, 1/8 and 1/6, but while one was sub-passaged, the other was not. This is unfortunate, since the isolate in question was O100, the same isolate which has recently been reported to transmit successfully to a macaque after a very protracted incubation period (Comoy et al., 2015).

Therefore, the first part of the study shows that the potential for scrapie transmission in tg mice is: i) low (based on tg650) or absent (based on tg 340) in MM129 mice; ii) low or absent in MV129 mice; iii) absent in VV129 mice. It is also important to compare these results with those obtained with BSE (a positive control due to its known zoonotic potential) in this and previous studies. These data indicate that the transmission barrier appears to be high for Classical scrapie with all three codon 129 polymorphisms.

However, three isolates (MF17, PS21 and PS42) after three serial passages produced incubation periods (IP) of 200–300 days post-inoculation (dpi), which is not a very long IP for this species, and which falls well within the limit of the mouse lifespan.

At first passage there were no clinically positive mice and at second passage there were a small number of positive mice (see Table 2). A possible explanation could be that overcoming the transmission barrier at first passage resulted in a low PrP^{Sc} titre, which is reflected in the low attack rates and relatively long IP in the second passage. The IP of these strains can therefore be calculated in two ways: considering the incubation period observed at third passage (200–300 dpi) or the sum of the Ips at first, second and third passage (1 382–1 482 dpi minimum).

The second part of the study shows that upon adaptation in tgHu, scrapie isolates resulted in a disease phenotype similar or identical to sCJD MM1. These results however derived from the study of a rather limited set of disease phenotypes, i.e. incubation times, Western blot profiles of PrP^{Sc} and PET-blot analysis of PrP^{Sc} deposition. It is important to appreciate here the potential effect of the PrP sequence of the host on the observed disease phenotypes. The range of PrP^{Sc} conformations, which are considered to reflect prion strain diversity, might be limited for a given PrP sequence. Indeed Cassard et al. (2014) used this reasoning to explain why different scrapie strains resulted in the same

disease phenotype after transmission in tgHu. It is not clear why the same reasoning was not applied to provide an alternative explanation for the observed convergence of scrapie and sCJD MM1 in tgHu.

The discussion of the paper focuses mostly on the possibility of a causative link between scrapie and sCJD. The results obtained, in combination with the data already known from literature; do not provide evidence for such a link. The paper raises the possibility that scrapie prions have the potential to be zoonotic, but does not provide evidence that transmission can or does take place under field conditions.

This paper also offers comparison of the biological properties of scrapie and sCJD after adaptation in tgHu mice. However, many of the conclusions in the paper are based on the results of serial passages. Analysis of the biological and biochemical properties of affected mice at each passage would have offered an insight into whether there were phenotypic changes, particularly between first and second passage, that would support or refute the suitability of using sub-passaged isolates for phenotypic comparison.

The artificial set-up of serial passages of scrapie isolates in transgenic mice should not be underestimated. In particular, the influence of the host genotype on the outcome of an isolate is not yet clarified. For example most of the scrapie isolates propagate in tgMet129 only and reflect the phenotype of sCJD in these tgHu after second passage. The authors mention the strain convergence theory, which results in similar prion strains after interspecies transmission. In this regard the emergence of sCJD is not so surprising. Even after inoculation of BSE, sCJD can emerge (Asante et al., 2001; Beringue et al., 2008b). There might be a limited diversity of prion strains in tgHu mice and/or the scrapie strains used are possibly not as stable as BSE. It should also be noted that while the different scrapie isolates act differently from each other in the analyses that contributed to their initial selection, there appears to be no phenotypic distinction between them in the final analysis in the tgHu models.

While these data would suggest a conclusion pointing to the low zoonotic potential of some scrapie strains, the main conclusion of the paper also suggests a direct link between Classical scrapie and sCJD. Following the reasoning in the discussion, it is implied that the most important data supporting a direct link derive from sub-passages in tgHu mice and from the comparison of the biological characteristics of these isolates with those of human prions in the same tgHu mice. In particular, Cassard et al. (2014) seem to use the biological comparison to speculate on zoonotic potential.

It is potentially misleading to interpret the similarity between scrapie and sCJD isolates in these mice as being indicative of zoonotic potential without independent supportive parallel information, for example trace back from human cases into a genetically unrelated model (e.g. bovine or porcine transgenes, or voles) to look for similarity against a different background, although strain convergence could still occur and may interfere with the interpretation of data, or most importantly strong epidemiological evidence.

Table 2: Overview of the most important BSE/scrapie tgHu mouse bioassays as in Cassard et al. (2014)

TSE agent strain	tg340 mice		tg340/tg361 F1 cross mice		tg361 mice		tg340, tg361 and their F1 cross mice						tg650 mice	
	1st ↓ M/M	2nd M/M(a) ↓ M/M	1st ↓ M/V	2nd M/V(a) ↓ M/V	1st ↓ V/V	2nd V/V(a) ↓ V/V	2nd M/M(b) ↓ M/M	2nd M/M(b) ↓ V/V	3rd M/M ↓ M/MI ↓ M/M	3rd M/M ↓ M/MI ↓ V/V	3rd M/V ↓ M/VI ↓ M/M	3rd M/V ↓ M/VI ↓ V/V	1st ↓ M/M	2nd M/M(a) ↓ M/M
Ovine scrapie MF17	Neg		**2/6**	3/6	Neg	Neg					6/6	6/6		
Ovine scrapie PS09	Neg	1/6	Neg	Neg	Neg	Neg								
Ovine scrapie PS21	Neg	2/6	Neg	Neg	Neg	Neg			6/6	6/6			**1/8**	11/11
Ovine scrapie PS48	Neg	Neg	Neg	Neg	Neg	Neg							Neg	
Ovine scrapie PS42	Neg	1/6	Neg	Neg	Neg	Neg			6/6	6/6				
Ovine scrapie PS310	Neg		Neg		Neg								Neg	Neg
Bovine BSE	**1/6**	6/6	Neg		Neg	Neg								
Bovine BSE	Neg	3/6	Neg		Neg	Neg								
Bovine BSE(d)													**2/6(d)**	6/7(d)
Human sCJD MM1	**6/6**	6/6	**6/6**	6/6	**6/6**	6/6	6/6	6/6					**6/6**	4/4
Human sCJD VV2	**6/6**	6/6	**6/6**	6/6	**6/6**	6/6	6/6	6/6					**6/6**	6/6
Human vCJD	**6/6**	6/6	**2/6**	6/6	Neg	Neg							**8/8**	7/7
Ovine scrapie 0100													**1/6**	

1st/2nd/3rd: number of passage in the mouse line; ↓: indicates the mouse line of the following passage.

M/M = transgenic mice that express the Met/Met_{129} human PrP; V/V = transgenic mice that express the Val/Val_{129} human PrP; M/V = crossbreed of transgenic mice that express Met_{129} and Val_{129} human PrP; $tg650_0$: transgenic mice that express the Met_{129} human PrP.

Neg = negative result in mouse bioassay; positive results are shown with number of positive mice/total number of mice examined; shaded cells indicate that mouse bioassays were not done.

BSE: bovine spongiform encephalopathy; sCJD: sporadic Creutzfeldt-Jakob disease; tgHu: humanised transgenic mice (expressing human PrP); vCJD: variant Creutzfeldt-Jakob disease.

(a): After first passage clinically affected or asymptomatic mice that survived >500 days post infection were pooled and used for second passage in the same line.
(b): Not clearly stated if the brain material which was used for inoculation was from one clinically affected mouse or from pooled brain material.
(c): The brain from one clinically affected mouse per isolate was used to inoculate fresh groups of mice.
(d): It is not indicated whether this bovine BSE isolate is the same as one of the two isolates used in the other experiments.

3.1.3. Concluding remarks

- The paper by Cassard et al. (2014) studies the effect of the Met/Val codon 129 polymorphism on the susceptibility of tgHu mice by inoculating a panel of scrapie isolates which represents at least a proportion of Classical scrapie strain diversity. By definition this approach cannot directly address all of the parameters needed to make a definitive assessment of zoonotic potential.
- The paper provides important new information on the aspects of the transmission barrier of Classical scrapie isolates in humans and indicates that no absolute transmission barrier limits the propagation of scrapie in tg mice expressing human MM129 or MV129 PrP, at least at the molecular level. The robustness of the results is enhanced in that similar outcomes are observed in three different laboratories with two different transgenic lines, minimizing the possibility of any confounding effect or possible cross-contamination.
- Although over-expression of PrP is not a natural condition in any species, the use of over-expressing transgenic mice can be considered a scientifically appropriate approach for modelling the molecular barrier for transmission of scrapie to humans.
- This study makes a rational choice of isolates designed to be distinct from one another, and to represent a range of field strains.
- Intracerebral inoculation is a widely accepted and appropriate choice of method to assess the permeability of the transmission barrier at the molecular level. However, this inoculation route cannot reproduce field conditions.
- The first part of the study shows that the potential for scrapie transmission in tg mice is: i) low or absent in MM129 mice; ii) low or absent in MV129 mice; iii) absent in VV129 mice. The data suggest that BSE is still more efficient than scrapie in MM129 mice, while a single scrapie isolate would be more efficient than BSE in MV129 mice.
- After serial sub-passages in transgenic mice, some of the scrapie isolates resulted in a sCJD-like disease with a high attack rate in recipient mice expressing MM129 and MV129 human PrP. The authors considered this finding as supportive of a potential causal link between scrapie and sCJD, but the results obtained, in combination with the data already known from literature, do not convincingly support such a theory. A more profound discussion of that matter is missing, in particular discussion of those aspects which might be less supportive of a causal link.
- A switch of disease phenotypes after serial passages *in vivo* or *in vitro* has been described before, even a switch to sCJD. This could be an alternative explanation for the results observed by Cassard et al. (2014) rather than a causative link between scrapie and sCJD. Phenotypic data on the BSE control isolates in this model would have been helpful in resolving this question.
- Overall, the paper provides good information regarding the choice of methods and protocols, in particular the animal models and inocula used. The results are thoroughly described and the species barrier issues are addressed. The paper raises the possibility that scrapie prions have the potential to be zoonotic, but does not provide evidence that transmission can or does take place under field conditions.

3.2. Review of the conclusions of the former EFSA-ECDC Opinion (EFSA BIOHAZ Panel, 2011)

In 2011, EFSA and ECDC published a 'Joint Scientific Opinion on any possible epidemiological or molecular association between TSE in animals and humans' (EFSA BIOHAZ Panel, 2011).

In response to ToR 2 of the mandate, this current Opinion reviews both the paper by Cassard et al. (2014) (see Section 3.1) and the literature (2010 – present day) on studies relevant to the assessment of the zoonotic potential of Classical and Atypical scrapie (see Section 1.4) and updates, where relevant, the conclusions of the former EFSA-ECDC Opinion (BIOHAZ Panel, 2011).

In brief, the 2011 Opinion highlighted that active surveillance has identified three new forms of animal TSE (L-BSE, H-BSE and Atypical scrapie), but that the information obtained had major limitations due to the unknown sensitivity of the current monitoring system for these TSE. There was no epidemiological evidence to suggest that Classical scrapie is zoonotic, and insufficient data to conclude whether or not the Atypical scrapie agent has zoonotic potential. However, experimental data suggested that the L-BSE agent does have significant zoonotic potential, which appeared 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 were extremely limited or not yet available, a single study reported efficient transmission of a natural sheep Classical scrapie isolate to primates. On the other hand, no direct scientific evidence of scrapie being a zoonotic disease had been presented. In a previous Opinion, the EFSA BIOHAZ Panel (2007) concluded that there was no evidence for an epidemiological or molecular link between classical and/or Atypical scrapie and TSE in humans, with BSE being the only TSE agent identified as zoonotic. However, the transmissibility of other animal TSE agents to humans could not be excluded.

In the absence of direct evidence of transmission to man, one definition of a zoonotic agent is that it fulfils the Bradford-Hill criteria (EFSA BIOHAZ Panel, 2011; see Table 3). If the criteria are only partially met then the possibility that an agent is zoonotic cannot be dismissed, but there is insufficient data to support its unequivocal classification as zoonotic.

Experimental evidence has to be balanced by the human epidemiological evidence, which has not demonstrated that Classical scrapie is zoonotic. As with all epidemiological data the epidemiological observations cannot exclude the possibility that animal TSE transmit rarely to humans and it is important to maintain vigilance.

Most of the conclusions from the former ECDC-EFSA 'Joint Scientific Opinion on any possible epidemiological or molecular association between TSE in animals and humans' (EFSA BIOHAZ Panel, 2011) remain valid at present; only four require minor amendments.

In the 2011 ECDC-EFSA Joint Scientific Opinion the Bradford-Hill criteria were used for judging the strength of evidence of causation in a zoonotic disease. In that opinion only two out of nine of the Bradford-Hill criteria, 'plausibility' and 'analogy', were judged to be fulfilled for Classical scrapie and Atypical scrapie. The new data from Cassard et al. (2014) and Comoy et al. (2015) enable the modification of the score for the 'experiment' criterion to positive for Classical scrapie (see Table 3).

The new scientific evidence available since 2010 supports and strengthens the conclusions of the 2011 ECDC-EFSA Joint Scientific Opinion with regard to the potential for some animal TSE to be zoonotic, but does not provide evidence of a causal link between animal TSE (including Classical and Atypical scrapie) and human TSE, with the exception of Classical BSE and vCJD.

Table 3: Updated assessment of putative links between animal and human TSE 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	+	+	+		+/-	+[I]	
9. Analogy	+	+	+	+	+	+	+

+: some scientific evidence is available for a positive interpretation; +/-: debatable or conflicting evidence is available;
BSE: bovine spongiform encephalopathy; CWD: Chronic Wasting Disease; H-BSE: H-type BSE; L-BSE: L-type BSE.

(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): scoring change compared the judgement by the EFSA BIOHAZ Panel (2011) according to the new evidence provided by Cassard et al. (2014) and Comoy et al. (2015).

In the context of the review of the data produced since 2010, the following conclusions from the 2011 ECDC-EFSA Opinion (EFSA BIOHAZ Panel, 2011) remain valid:

- *'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.*
- *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.*
- *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.*
- *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*.'*

The remaining four conclusions from the 2011 ECDC-EFSA Opinion (EFSA BIOHAZ Panel, 2011) require some modifications. The former conclusions are listed below, each one of them followed by the new version, revised in light of the new scientific evidence available:

- Former conclusion: '*Except for Classical BSE, there is limited epidemiological information on the prevalence and distribution of individual ruminant TSE agents.*', revised as follows:

  Except for Classical BSE, there is limited epidemiological information on the prevalence and distribution of individual ruminant TSE agents. However, based on data from the European active surveillance since 2002, the knowledge of the geographical distribution of Classical scrapie and Atypical scrapie has improved. Whereas Atypical scrapie shows low variability between countries, the prevalence of Classical scrapie is highly heterogeneous and within-

country data are not readily available. With regard to Classical scrapie there is no information on the distribution of individual strains.

- Former conclusion: '*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).*', revised as follows:

  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, Classical BSE in sheep and some Classical scrapie isolates) might have zoonotic potential, whereas for other agents there is no evidence provided of zoonotic potential (H-type Atypical BSE, Atypical scrapie and CWD agents).

- Former conclusion: '*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.*', revised as follows:

  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, some Classical scrapie isolates, TME, CWD agents) might have zoonotic potential. In particular, primates are highly permissive to L-type Atypical BSE, even by the oral route.

- Former conclusion: '*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.*', revised as follows:

  While transmission data for evaluating the zoonotic potential of Classical scrapie in primates and human PrP transgenic mice are limited, two studies reported transmission of Classical scrapie isolates to primates and one to transgenic humanised mice.

4. Conclusions

4.1. General conclusions

- There is no evidence of an absolute species barrier. There are many factors that influence the ability of any TSE agent to infect a host, regardless of whether the infection occurs across a species barrier. For example, exposure to a sufficient dose of the agent, uptake of the agent from the gastrointestinal tract and neuroinvasion and successful conversion of PrP^{C} to PrP^{Sc} are all required. It is impossible to define an experimental model that encompasses this potential variability and that directly measures zoonotic potential.
- Several distinct forms of prion disease have been described in humans and animals. Some are genetic in origin, and some are acquired. Creutzfeldt-Jakob disease, which represents around 90 % of human prion cases, occurs globally in several forms. Sporadic CJD (sCJD), which can be classified into six subtypes, is the most common. Scrapie in small ruminants can also be classified in several distinct strains.
- Surveillance of sCJD has been carried out in the majority of Member States for many years. Although there is variation from year to year, the mean overall annual mortality rates are relatively consistent at 1–1.5 cases per million, suggesting that should there be an environmental risk factor for the development of disease, this must be ubiquitous and evenly distributed. The absence of any consistent risk factor in sCJD, including occupation and dietary exposures, together with the epidemiological data, is compatible with the hypothesis that the majority of cases of sCJD arise as a spontaneous disease with no external source of infection.

4.2. Answer to Term of Reference 1

- The paper under appraisal ('Evidence for zoonotic potential of ovine scrapie prions', by Cassard et al., 2014) uses a combination of intracerebral inoculation, transgenic mice overexpressing human prion protein and serial passages that maximises the chance of detecting the propagation of TSE agents, but does not mimic natural exposure.
- The paper provides evidence in a laboratory experiment that some Classical scrapie isolates can propagate in humanised transgenic mice and produce prions that on second passage are similar to those causing one form of sCJD.
- The paper raises the possibility that scrapie prions have the potential to be zoonotic, but does not provide evidence that transmission can or does take place under field conditions.

4.3. Answer to Term of Reference 2

- Most of the conclusions from the former 2011 ECDC-EFSA 'Joint Scientific Opinion on any possible epidemiological or molecular association between TSEs in animals and humans' (EFSA BIOHAZ Panel, 2011) remain valid at present; only four require minor amendments.
- In the 2011 ECDC-EFSA Joint Scientific Opinion the Bradford-Hill criteria were used for judging the strength of evidence of causation in a zoonotic disease. In that opinion only two out of nine of the Bradford-Hill criteria, 'plausibility' and 'analogy' were judged to be fulfilled for Classical scrapie and Atypical scrapie. The new data from Cassard et al. (2014) and Comoy et al. (2015) enable the modification of the score for the 'experiment' criterion to positive for Classical scrapie.
- The new scientific evidence available since 2010 supports and strengthens the conclusions of the 2011 ECDC-EFSA Joint Scientific Opinion with regard to the potential for some animal TSE to be zoonotic, but does not provide evidence of a causal link between Classical and Atypical scrapie and human TSE.

4.4. Answer to Term of Reference 3

- The specific biochemical and biological factors that might determine zoonotic potential have not been identified, but there is no evidence of an absolute species barrier.
- Recent publications have demonstrated that some Classical scrapie prions have the potential to convert human or primate PrP to the disease-associated form in *in vivo* experimental models, but do not allow assessment of the risk under field conditions.
- A spectrum of strains is responsible for Classical scrapie in sheep, and there may be variability in properties that affect the ability to cross the species barrier. There is experimental evidence that some isolates may not be completely stable, and their fundamental properties may shift on transmission. There is also potential heterogeneity of geographical distribution of individual strains.
- From the available epidemiological evidence it is not possible to conclude that the exposure of consumers to ovine products has resulted in the transmission of prion diseases to humans, nor that Classical scrapie is zoonotic.
- The level of exposure of consumers to scrapie agents through ovine products is largely determined by the prevalence of the disease in ovines and by the amount of infectivity in ovine tissues entering the food chain. The latter is reduced by the current SRM measures.
- A quantitative assessment of the overall amount of infectivity from TSE-infected ovine products entering the food chain would require data on infectivity distribution in small ruminants, disease frequency at population level, and sensitivity of detection of TSE-infected small ruminants at slaughter, among others. Data on these parameters and a mathematical model developed and applied previously by EFSA could be used for this purpose. An individual exposure to scrapie agents could be assessed only by combining the results from such an assessment with additional information such as consumption data and ovine product information.

- Current evidence does not establish a causal link between scrapie and sCJD. The possibility of scrapie-related public health risks from the consumption of ovine products cannot be assessed.

5. Recommendations

The BIOHAZ Panel recommends:

- further investigations on the variability of scrapie strains in field conditions and on their zoonotic potential;
- further experimental work to determine the biochemical and biological parameters that influence cross-species transmission of TSE diseases and related barriers;
- continued and improved surveillance of animal and human TSE to monitor the evolution of the diseases and to allow their epidemiological comparisons and investigate their associations in the future;
- a quantitative assessment of the overall amount of infectivity from TSE-infected ovine products entering the food chain: a mathematical model developed in the framework of a previous EFSA opinion could be adapted for this purpose;
- inclusion of caprine products in any assessment of scrapie exposure;
- collection of data on consumption patterns and product information of food products originating from small ruminants to enable an exposure assessment at individual level.

Documentation provided to EFSA

1. Cassard H, Torres JM, Lacroux C, Douet JY, Benestad SL, Lantier F, Lugan S, Lantier I, Costes P, Aron N, Reine F, Herzog L, Espinosa JC, Beringue V and Andreoletti O, 2014. Evidence for zoonotic potential of ovine scrapie prions. Nature Communications, 5, 5821.

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Abbreviations

AS	Atypical scrapie
BSE	bovine spongiform encephalopathy
C-BSE	Classical BSE
CISA	Centro de Investigacion en Sanidad Animal
CJD	Creutzfeldt-Jakob Disease
CNS	central nervous system
CS	Classical scrapie
CWD	chronic wasting disease
CWRU	Case Western Reserve University
dpi	days post-inoculation
ECDC	European Centre for Disease Prevention and Control
ENS	enteric nervous system
EU	European Union
EU-27	European Union (excluding Croatia)
EUROCJD	European Creutzfeldt-Jakob Disease International Surveillance Network
fCJD	familial Creutzfeldt-Jakob disease
FFI	fatal familial insomnia
FSE	feline spongiform encephalopathy
GALT	gut-associated lymphoid tissue
gBSE	goat BSE
GSS	Gerstmann-Sträussler-Scheinker
H-BSE	H-type BSE
i.c.	intracerebral
iCJD	Iatrogenic Creutzfeldt-Jakob Disease
INRA	Institute National de la Recherche Agronomique
i.p.	intraperitoneal
IP	incubation period
L-BSE	L-type atypical BSE
LRS	lymphoreticular system
n/a	Not available
oBSE	ovine BSE
PK	Proteinase K
PMCA	protein-misfolding cyclic amplification
PP	Peyer's Patches
PrP	prion protein
PrP^{C}	cellular PrP

PrP^{Res}	protease-resistant form of PrP, also used as synonymous of PrP^{Sc}
PrP^{Sc}	abnormal form of PrP, also used as synonymous of PrP^{res}
QuIC	quaking-induced conversion
sCJD	sporadic Creutzfeldt-Jakob Disease
SRM	specified risk material
tg	transgenic
tgBov	bovinized transgenic mice (expressing bovine PrP)
tgHu	humanised transgenic mice (expressing human PrP)
TME	transmissible mink encephalopathy
ToR	Terms of reference
TSE	transmissible spongiform encephalopathy
vCJD	variant Creutzfeldt-Jakob Disease
VPSPr	variably protease-sensitive prionopathy

Appendix A – Comparison with results from other scientific publications

A number of different transgenic mouse lines expressing human PrP have been produced and used to assess the molecular permeability of the transmission barrier of animal TSE to humans. The comparison of the reported results, including those reported by Cassard et al. (2014), is not straightforward, as several technical considerations have to be evaluated when comparing these types of studies. These include, but are not limited to the following:

- type of transgene used;
- level and site of PrP expression;
- human PrP variant expressed;
- inoculation site and dose;
- sampling and diagnostic procedures;
- duration of the experiments;
- infectious titre;
- choice of the individual animal TSE isolates to test.

In Tables 4–6, some of these aspects such as expression level of PrP and inoculation dose have been considered but others were not easily assessable. Thus, the considerations reported below cannot be seen as an exhaustive assessment on the transmissibility of animal TSE in transgenic mouse lines expressing human PrP, but rather represent a guide to critically review the available evidence.

Preliminary considerations

- As the 129M allele of the human PrP is associated with susceptibility to BSE and vCJD, the transmissibility of animal TSE has been focused in five transgenic mouse lines overexpressing 129M PrP on a mouse PrP null background, and a gene-targeted mouse line where the native mouse PrP was replaced by the human 129M PrP. By comparison, fewer experiments have been performed in genetically modified mouse lines expressing human 129V. In addition there are difficulties generating overexpressing heterozygotes, i.e. 129MV, as two lines, one 129MM and one 129VV, with equal overexpression levels are required. Such lines are very difficult to source, as it is not possible to control transgene expression levels in advance. This is not an issue with gene-targeted models as by design they express similar levels of the transgene. The study by Cassard et al. (2014) is therefore unique in this respect, as two different lines expressing similar levels of 129MM and 129VV have been used allowing the generation of 129MV overexpressing mice. It therefore covers the full spectrum of allelic variability regarding this codon of the human PrP.
- Positive mice as reported in the tables include animals confirmed TSE positive using one of the available diagnostic methods (histopathology, Western blot, immunohistochemistry or any combination of these), irrespective of clinical status. However, the diagnostic procedures may have different sensitivity in different laboratories, which may affect experiments with very low attack rates. In such cases successful transmission may depend on detection of minimal diagnostic threshold levels.
- Only attack rates are presented in the tables as they represent the most reliable indicator of transmission efficiency.
- For the studies included in the tables, the results obtained with one or more inocula of a given TSE are summarised to give an overall attack rate for the TSE under consideration. This approach is more appropriate when it is applied in single strain TSE. However, the existence of multiple Classical scrapie strains indicates that there may be biological variability among different Classical scrapie sources. Therefore where successful transmissions of Classical scrapie are summarised in the tables the results of individual inocula are made explicit in the notes.
- As sCJD consists of different pathological types, the results are summarised to produce an overall attack rate for sCJD ignoring the sCJD type.

- Blind second passages can aid in detecting sub-clinical infections even when after primary transmission no positive mice were detected. So far this approach has been used in a limited number of experiments, which are included in the tables. It cannot be excluded that subclinical infections, not detectable by standard diagnostic procedures, may have occurred in experiments where blind second passages were not performed.
- In cases where there is a strong molecular transmission barrier between source and challenged experimental model the attack rates are expected to be low. In these cases small scale experiments, which typically involve challenges of 6-10 animals with a single source, may not reveal the full extent of the permeability of specific barriers if they are viewed in isolation, particularly when they generate negative transmission data. For example a Classical scrapie source that transmitted on first passage to tg650 mice failed to do so in tg340 mice. However, after serial passaging it transmitted to both mouse lines. This may cast doubts on the overall transmissibility of other sources that may have failed to transmit during small scale experiments.

Human Inocula

The results obtained with sCJD and vCJD cases are included in the tables in order to show the reliability of the transgenic mouse lines in reproducing the human species barrier, as human inocula are expected to transmit easily in mice expressing appropriate human PrP variants. Indeed, sCJD cases were transmissible with high attack rates to all transgenic mouse lines considered, expressing 129MM PrP, 129VV PrP or 129MV PrP. In contrast, vCJD cases which are all 129MM transmitted efficiently only in transgenic mice expressing 129MM PrP, while they transmitted less efficiently in transgenic mice expressing 129MV PrP and with low efficiency or not at all in those expressing 129VV PrP.

Overall, the results obtained with human TSE suggest that the transgenic mouse lines considered can reproduce the expected molecular transmission barriers to human inocula.

Bovine BSE (C-BSE)

As the only known animal TSE with definite zoonotic potential, C-BSE can be considered a benchmark for evaluating the zoonotic potential of other animal TSE. Since vCJD infections were only reported in humans homozygous 129MM PrP, it is expected that C-BSE should be transmissible to recipient 129MM mice, but might transmit less efficiently or not at all in those carrying 129MV PrP or 129VV PrP genotypes. Indeed, with the exception of the gene-targeted tgMM line, C-BSE transmitted in all recipient mouse lines overexpressing 129MM PrP, while it transmitted rather inefficiently in a single 129VV mouse line and did not transmit in any 129MV mouse lines. Variable results in mice expressing 129VV PrP might depend on the level of transgene expression although other factors cannot be excluded and emphasize the impact of the mouse line used on the outcomes of the experiments.

The efficiency of transmission in transgenic mice expressing 129MM human PrP was rather variable according to the different mouse lines. This can be attributed only partially to the level of transgene expression in the recipient mouse lines. While no transmission was observed in the gene-targeted 129MM mice, which express physiological levels of PrP, the efficiency of transmission does not seem to correlate well with PrP expression levels in overexpressing mouse lines. Of note, blind second passages in 129MM PrP mouse lines having low attack rate at primary transmission resulted in high rate of transmission, suggesting the presence of subclinical infection in negative mice after primary transmission.

Small ruminant C-BSE

Overall, experiments with small ruminant BSE mirrored those with cattle BSE in that transmission was observed in 129MM recipient mice, but not in those expressing 129VV or 129MV PrP. In several 129MM PrP mouse lines the transmission efficiency of small ruminant BSE was even higher than that of cattle BSE, but there were exceptions, again emphasizing how the use of different mouse lines might have impact on the final outcome irrespective of the transgene expressed or the level of expression.

Overall, the results obtained with small ruminant BSE suggest that this animal TSE could have the potential to propagate in humans carrying 129MM PrP genotype.

Classical scrapie

Classical scrapie isolates have been inoculated in the transgenic mouse lines reported in the tables, with the exception of tg40 expressing 129MM PrP genotype. The results were negative in the majority of cases. However, Cassard et al. (2014) reported positive transmissions with low attack rates in 129MM and in 129MV recipient mice. Comparison with cattle BSE in tg650 expressing 129MM PrP reveals similar attack rates between bovine BSE and two scrapie isolates that resulted in successful transmissions. However, blind second passages in this line resulted in an overall attack rate lower than that of cattle BSE.

Overall, these results suggest that a transmission barrier limits the transmissibility of most scrapie isolates in mice expressing human PrP. However, the data also show that this barrier is not absolute, and specific Classical scrapie isolates may transmit as efficiently as cattle BSE. Given the strain variability observed in field cases of scrapie, these results are important as they might indicate some variation in the zoonotic potential among different scrapie strains.

Atypical scrapie

Experiments with Atypical scrapie are limited, having been attempted in only two overexpressing lines, one expressing 129MM PrP and one expressing 129VV PrP. In gene-targeted mice Atypical scrapie transmission has been attempted in 129MM, 129MV and 129VV PrP backgrounds. No positive transmissions have been observed in any of these experiments, suggesting a high molecular barrier for propagation of Atypical scrapie in mice expressing human PrP.

L-BSE

Experiments with L-BSE are limited having been reported in only two overexpressing mouse lines, both carrying 129M transgenes. In gene-targeted mouse lines, challenges in 129MV and 129VV in addition to the 129MM have been attempted. No positive transmission has been reported in any gene-targeted mouse lines, while significant transmission efficiency has been reported in both mouse lines overexpressing 129MM PrP. The transmission efficiency observed in tg650 mice overexpressing 129MM PrP was even higher than that observed for C-BSE in the same mouse line.

Overall, although the data available L-BSE are still limited, results obtained in a tg650 mice suggest that the molecular barrier of human PrP to L-BSE could be lower compared to C-BSE, indicating a possible zoonotic potential for L-BSE.

H-BSE

Similarly to L-BSE, experiments with H-BSE are limited having been reported in only two overexpressing mouse lines, both carrying 129M transgenes. In gene targeted mice, challenges in 129MV and 129VV in addition to the 129MM have been attempted. In this limited number of experiments none of the challenges resulted in a positive transmission suggesting a higher barrier compared to C-BSE.

CWD

Isolates of CWD have been inoculated in six different transgenic mouse lines, in both gene-targeted (tgMM, tgMV and tgVV) and overexpressing 129MM (tg35, tg40) or 129VV (tg152) genotypes. No positive transmissions have been observed in these experiments, suggesting a high molecular barrier for propagation of CWD in mice expressing human PrP.

Table 4: Transmission of human and animal TSE in mouse lines expressing human 129MM PrP

Passage, mouse line and expression level			Reference	sCJD	vCJD	C-BSE	oBSE	gBSE	CS	AS	L-BSE	H-BSE	CWD	Inoc. route	Dose	Survival
1st	tgMM	1x	Bishop et al. (2006)		**11/17**	0/18								i.c.	20 µl, 10 %	Life
1st	tgMM	1x	Bishop et al. (2010)	**56/88**										i.c.	20 µl, 10 %	Life
1st	tgMM	1x	Plinston et al. (2011)				**17/43**		0/48					i.c.	20 µl, 10 %	Life
1st	tgMM	1x	Wilson et al. (2012)[(b)]							0/164	0/24	0/24	0/24	i.c.	20 µl, 10 %	Life
1st	tgMM	1x	Wilson et al. (2012)[(b)]			0/14					0/58			i.c. and i.p.	20 µl and 100 µl, 10 %	Life
1st	tgMM	1x	Wilson et al. (2013)[(c)]					**10/24**	0/45[(c)]					i.c.	20 µl, 10 %	Life
1st	tg35	2x	Asante et al. (2002)	**44/50**	**14/14**	**14/49**								i.c.	30 µl, 1 %	Life
1st	tg35	2x	Sandberg et al. (2010)										0/9	i.c.	30 µl, 1 %	Life
1st	tg35c [(a)]	2x	Wadsworth et al. (2013)	**9/9[(d)]**	**12/12**	**5/12**	**1/35**		0/86	0/66				i.c.	30 µl, 1 %	Killed at 600–700 dpi
1st	tg45	4x	Asante et al. (2002)		**4/4**	**9/12**								i.c.	30 µl, 1 %	Life
1st	tg40	1x	Kong et al. (2005, 2008)	**19/19**							**18/3**		0/29	i.c.	30 µl, 1 %	Life
1st	tg650	6x	Beringue et al. (2008b)	**30/30**	**38/57**									i.c.	20 µl, 10 %	Life
1st	tg650	6x	Beringue et al. (2008a)			**4/25**					**33/3**	0/22		i.c.	20 µl, 10 %	Life
1st	tg650	6x	Padilla et al. (2011)		**37/41**	**4/25**	**10/11**	**11/11**						i.c.	20 µl, 10 %	Life
1st	tg650	6x	Cassard et al. (2014)	**12/12**	**8/8**	**2/6**			**2/29[(e)]**					i.c.	20 µl, 10 %	Life
1st	tg340	4x	Padilla et al. (2011)	**5/5**	**6/6**	**1/15**	**4/6**	**6/7**						i.c.	20 µl, 10 %	Life
1st	tg340	4x	Torres et al. (2014)	**5/5**	**6/6**	**1/8**	**6/6**		0/6			0/6		i.c.	20 µl, 10 %	Life
1st	tg340	4x	Cassard et al. (2014)	**12/12**	**6/6**	**1/12**			0/36					i.c.	20 µl, 10 %	Life
2nd	tg650	6x	Cassard et al. (2014)	**10/10**	**7/7**	**6/7**			**11/36[(f)]**					i.c.	20 µl, 10 %	Life
2nd	tg340	4x	Cassard et al. (2014)	**12/12**	**6/6**	**9/12**			**4/24**					i.c.	20 µl, 10 %	Life
2nd	tg340	4x	Torres et al. (2014)	**6/6**	**6/6**	**4/4**	**5/5**		0/6			0/6		i.c.	20 µl, 10 %	Life

1st/2nd: number of passage in the mouse line.

AS = Atypical scrapie; BSE: bovine spongiform encephalopathy; C-BSE: classical BSE; CS = Classical scrapie; CWD: chronic wasting disease; dpi: days post-inoculation; gBSE = goat BSE; H-BSE: H-type BSE; i.c.: intracerebral; inoc. route = inoculation route; i.p.: intraperitoneal; L-BSE: L-type atypical BSE; oBSE = ovine BSE; sCJD: sporadic Creutzfeldt-Jakob disease; TSE: transmissible spongiform encephalopathy; vCJD: variant Creutzfeldt-Jakob disease.

Bold: positive transmission.

(a): mouse line congenic with tg35 rederived on a FVB/N background.
(b): study conducted in different laboratories applying different inoculation protocols.
(c): two isolates of goat scrapie, including one with some BSE-like properties.
(d): Iatrogenic CJD from MM129 patient with Type 2 PrP^{Sc} by the London classification.
(e): positive mice are from two isolates giving an attack rate of 1/8 and 1/6, while two other isolates gave no transmission.
(f): only one isolate with 100 %, with second passage from individual positive brain.

Table 5: Transmission of human and animal TSE in mouse lines expressing human 129MV PrP

Passage, mouse line and expression level			Reference	sCJD	vCJD	C-BSE	oBSE	gBSE	CS	AS	L-BSE	H-BSE	CWD	Inoc. route	Dose	Survival
1st	tgMV	1x	Bishop et al. (2006)		**11/16**	0/17								i.c.	20 µl, 10 %	Life
1st	cross	1x	Bishop et al. (2010)	**61/95**										i.c.	20 µl, 10 %	Life
1st	cross	1x	Plinston et al. (2011)				0/47		0/47					i.c.	20 µl, 10 %	Life
1st	cross	1x	Wilson et al. (2012)[(a)]							0/163	0/24	0/24	0/24	i.c.	20 µl, 10 %	Life
1st	cross	1x	Wilson et al. (2012)[(a)]			0/15					0/66			i.c. and i.p.	20 µl and 100 µl, 10 %	Life
1st	cross	1x	Wilson et al. (2013)[(b)]					0/23	0/48[(b)]					i.c.	20 µl, 10 %	Life
1st	cross	4x	Cassard et al. (2014)	**12/12**	**2/6**	0/12			**2/36[(c)]**					i.c.	20 µl, 10 %	Life
2nd	cross	4x	Cassard et al. (2014)	**12/12**	**6/6**				**3/30**					i.c.	20 µl, 10 %	Life

1st/2nd: number of passage in the mouse line.

AS = Atypical scrapie; BSE: bovine spongiform encephalopathy; C-BSE: classical BSE; CS = Classical scrapie; CWD: chronic wasting disease; dpi: days post-inoculation; gBSE = goat BSE; H-BSE: H-type BSE; i.c.: intracerebral; inoc. route = inoculation route; i.p.: intraperitoneal; L-BSE: L-type atypical BSE; oBSE = ovine BSE; sCJD: sporadic Creutzfeldt-Jakob disease; TSE: transmissible spongiform encephalopathy; vCJD: variant Creutzfeldt-Jakob disease.

Bold: positive transmission.

(a): study conducted in different laboratories applying different inoculation protocols.
(b): two isolates of goat scrapie, including one with some BSE-like properties.
(c): positive mice are from a single isolate giving an attack rate of 2/6, while five other isolates failed to transmit.

Table 6: Transmission of human and animal TSE in mouse lines expressing human 129VV PrP

Passage, mouse line and expression level			Reference	sCJD	vCJD	C-BSE	oBSE	gBSE	CS	AS	L-BSE	H-BSE	CWD	Inoc. route	Dose	Survival
1st	tgVV	1x	Bishop et al. (2006)		**1/16**	0/22								i.c.	20 µl, 10 %	Life
1st	tgVV	1x	Bishop et al. (2010)	**77/95**										i.c.	20 µl, 10 %	Life
1st	tgVV	1x	Plinston et al. (2011)				0/46		0/46					i.c.	20 µl, 10 %	Life
1st	tgVV	1x	Wilson et al. (2012)[b]							0/163	0/24	0/24	0/24	i.c.	20 µl, 10 %	Life
1st	tgVV	1x	Wilson et al. (2012)[b]			0/13					0/56			i.c. and i.p.	20 µl and 100 µl, 10 %	Life
1st	tgVV	1x	Wilson et al. (2013)[c]					0/23	0/47[b]					i.c.	20 µl, 10 %	Life
1st	tg152	6x	Hill et al. (1997)	**86/87**	**25/56**	**10/26**								i.c.	30 µl, 1 %	Life
1st	tg152	6x	Sandberg et al. (2010)										0/9	i.c.	30 µl, 1 %	Life
1st	tg152c[a]	6x	Wadsworth et al. (2013)				0/35		0/86	0/66				i.c.	30 µl, 1 %	Killed at 600–700 dpi
1st	tg361	4x	Cassard et al. (2014)	**12/12**	0/6	0/12			0/36					i.c.	20 µl, 10 %	Life
2nd	tg361	4x	Cassard et al. (2014)	**12/12**	0/6	0/12			0/29					i.c.	20 µl, 10 %	Life

1st/2nd: number of passage in the mouse line.

AS = Atypical scrapie; BSE: bovine spongiform encephalopathy; C-BSE: classical BSE; CS = Classical scrapie; CWD: chronic wasting disease; dpi: days post-inoculation; gBSE = goat BSE; H-BSE: H-type BSE; i.c.: intracerebral; inoc. route = inoculation route; i.p.: intraperitoneal; L-BSE: L-type atypical BSE; oBSE = ovine BSE; sCJD: sporadic Creutzfeldt-Jakob disease; TSE: transmissible spongiform encephalopathy; vCJD: variant Creutzfeldt-Jakob disease.

Bold: positive transmission.

(a): mouse line congenic with tg152 rederived on a FVB/N background.
(b): study conducted in different laboratories applying different inoculation protocols.
(c): two isolates of goat scrapie, including one with some BSE-like properties.