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Animal Prion Diseases: the Risks to Human Health.

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Summary

Transmissible spongiform encephalopathies (TSEs) or prion diseases of animals notably include scrapie in small ruminants, chronic wasting disease (CWD) in cervids, and classical bovine spongiform encephalopathy (C-BSE). Due to the transmission barrier phenomenon that naturally limits the propagation of prions from one species to another, and the lack of epidemiological evidence for an association with human prion diseases, the zoonotic potential of these diseases was for a long time considered negligible.

However, in 1996 C-BSE was recognized as the cause of a new human prion disease, variant Creutzfeldt-Jakob disease (vCJD), which triggered an unprecedented public health crisis in Europe.

Large scale epidemio-surveillance programs for scrapie and C-BSE that were implemented in the EU after the BSE crisis revealed that the distribution and prevalence of prion diseases in the ruminant population had previously been underestimated. They also led to the recognition of new forms of TSEs (named atypical) in cattle and small ruminants and to the recent identification of CWD in Europe.

At this stage, the characterization of the strain diversity and zoonotic abilities associated with animal prion diseases remain largely incomplete. However, transmission experiments in non-human primates and transgenic mice expressing human PrP clearly indicate that classical scrapie, and certain forms of atypical BSE (L-BSE) or CWD may have the potential to infect humans. The remaining uncertainties about the origins and relationships between animal prion diseases emphasizes the importance of the measures implemented to limit human exposure to these potentially zoonotic agents, and of continued surveillance for both animal and human prion diseases.

Keywords: prion, TSE, BSE, scrapie, CWD, zoonotic

Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSE), are fatal neurodegenerative diseases in which a key feature of the pathogenesis is the accumulation of a misfolded form (PrP^{Sc}) of a normal host glycoprotein (PrP^{C}). The term prion (derived from **pr**oteinaceous **i**nfectious particle) arises from the hypothesis that infectious or contagious forms of these diseases are caused solely by transmission of PrP^{Sc} (108).

Animal prion diseases include scrapie in sheep, bovine spongiform encephalopathy (C-BSE) in cattle, chronic wasting disease (CWD) in cervids, and transmissible mink encephalopathy (TME) in farmed mink, which all have an infectious aetiology. The origin of new outbreaks or forms of animal prion disease are often obscure and therefore unpredictable, as evidenced by the recent emergence of CWD in Scandinavia and recognition of a novel prion disease of camels in Algeria (11, 17). In humans, as well as acquired prion diseases such as iatrogenic and variant Creutzfeldt-Jakob disease (iCJD, vCJD), there are genetic/inherited prion diseases, but the most common form of disease is sporadic CJD (sCJD) for which the aetiology is not fully understood (28, 57).

The first evidence for the infectious nature of prion diseases came from experiments in which scrapie was transmitted to healthy sheep by inoculation of brain extracts from a diseased animal (45, 46). Following this seminal result, experimental inoculation of scrapie and other prion diseases (kuru, Creutzfeldt-Jakob disease) was attempted in a variety of species, including many commonly used laboratory rodents, as well as non-human primates (58). It was quickly recognized that attempts to transmit infection from one species to another are limited by a transmission barrier, also named “species barrier”. Transmission barriers often result in a lack of propagation of prion in the new host species. In other cases, inoculation of a prion into a new host species produces a low or inconsistent disease incidence, and prolonged incubation periods or subclinical infection. After one or more sub-passages in the same host, the clinical incidence rate increases and incubation periods become shorter, and are ultimately very predictable for a defined dose and route of infection (21).

The molecular mechanisms that determine the permeability of transmission barrier to a prion in a particular host are still not fully understood. However, there is now a wealth of evidence that important factors influencing cross-species transmission include the nature of the prion strain, compatibility between the primary amino acid sequence of donor and host PrP, and the

dose/exposure route in the recipient host. Despite this progress, it still remains impossible to predict *a priori* the capacity of a prion to propagate in a new host species.

Concerns and speculation about the possibility of cross-species transmission of animal prion diseases to humans have existed since the infectious nature of these diseases was demonstrated. It has been known for several centuries that scrapie is endemic in sheep used for human food production (48). However, despite the likely exposure of certain individuals to infected sheep, no link could be established between scrapie and TSE occurrence in humans (132). This lack of evidence of zoonotic transmission led to the general opinion that a high transmission barrier protects humans from animal prion diseases. However, the emergence in 1996 of vCJD provided incontrovertible evidence that inter-species transmission barriers are not sufficient to protect the human population from prion agents circulating in domesticated animals and wildlife (139). The resulting public health crisis provided the impetus for development of novel experimental techniques and models, which have also been employed to estimate the risk to humans from other animal prion diseases. Using the example of BSE and vCJD, this paper will review and discuss the available evidence for associations between human and animal prion diseases.

Classical BSE and variant CJD

History and epidemiology

Classical BSE (C-BSE) was first recognized in 1984-85 as novel prion disease affecting cattle in the UK (136). The origin of C-BSE has still not been clearly established, but the number of cases was amplified by the recycling of infected carcasses into cattle feed in the form of meat and bone meal (MBM) (138).

BSE was disseminated to at least 28 countries, mostly in Europe but also in the USA, Canada, and Japan, through the export of infected live animals and/or contaminated MBM and livestock feed. However, the numbers of cattle affected were much lower than in the UK, with a total of 6,193 recorded cases in EU countries between 1989 and 2016.

In the UK, the C-BSE epidemic peaked in 1992, with more than 37 000 confirmed cases in that year (Figure 1). Legislation prohibiting the inclusion of MBM and other animal proteins in livestock feed in the UK (1988, 1996) and EU (2001) was instrumental in controlling transmission of BSE and exposure of other farmed animals. The incidence of C-BSE has now declined to very low levels, with no cases or a single-digit number of cases reported each year since 2011 (Table 1, Figure 1). It has been estimated that globally over 1 million BSE-infected cattle entered the human food chain, resulting in potential dietary exposure to C-BSE for millions of consumers [6]. Even if the number of C-BSE cases that occurred into the UK exceeded by several orders of magnitude those observed in other affected countries, international trade of food commodities could have resulted in exposure of people even in regions that did not experience autochthonous C-BSE cases.

The occurrence of a large epidemic of a novel prion disease in cattle rapidly raised concerns about the risk C-BSE might represent for humans. The first protective measures for the food chain were implemented in November 1989, with a ban on the use of certain specified bovine offal in human food (Figure 2). Concomitantly, the UK Department of Health set up the National CJD Surveillance Unit in 1990, whose mission was to monitor the incidence and study the epidemiology of human prion diseases.

The detection of a novel prion disease (feline spongiform encephalopathy) in cats in the UK, and the experimental transmission of the C-BSE agent to non-human primates (marmoset) further reinforced concerns regarding the ability of C-BSE to cross interspecies transmission barriers (1, 12). In 1995, two cases of CJD were reported in teenagers in the UK, an unusually early onset of disease (14, 27). These patients also displayed atypical clinical symptoms and distinctly different neuropathological changes compared to known human prion diseases, and the emergence of a new form of prion disease, named variant CJD (vCJD), naturally pointed to C-BSE as the probable causative agent (139).

Definitive evidence for the link between BSE and vCJD was provided by transmission studies in inbred mouse lines, in which mice injected with vCJD exhibited phenotypes (incubation periods and lesion profile) indistinguishable from those obtained following transmission of BSE or feline spongiform encephalopathy (FSE), indicating that all three diseases were caused by the same prion agent (32). Similarly, C-BSE isolates from individual affected cattle in different farms and locations displayed identical phenotypes following strain typing in mice, which confirmed that C-BSE was caused by a single prion strain (33, 38).

To date, out of 231 vCJD cases identified worldwide, 178 cases have occurred in UK residents, and a number of other cases have occurred in individuals with a history of residence in the UK during the high risk period 1980-1996 (http://www.cjd.ed.ac.uk/surveillance/data-and-reports). All definite clinical cases of vCJD that have undergone *PRNP* genetic analysis are homozygous for methionine at codon 129 (129MM), apart from the latest UK case, who was heterozygous (129MV) (95).

After a peak in 2001-2002, the number of vCJD cases recorded in the UK has declined. While the limited number of cases is consistent with inefficient transmission of the C-BSE agent to humans, many uncertainties remain concerning the number of individuals incubating vCJD in the exposed population (49). To address this issue, several studies have been performed to estimate the prevalence of vCJD infection in the UK population. Since abnormal PrP deposits (PrP^d) can be detected in the lymphoid organs of vCJD patients during preclinical and clinical phases of the disease (64, 106), prevalence studies were based on anonymized surveys of more than 45,000 appendix and tonsil samples removed and archived following routine surgery. Targeted patients belonged to age cohorts most likely to have experienced dietary exposure to C-BSE, and the tissues were tested for the presence of PrP^d by immunohistochemistry (IHC).

In two separate surveys, a total of 19 appendix samples were positive for abnormal PrP accumulation (59, 67). Strikingly, positive samples were found to have codon 129 methionine-methionine, methionine-valine and valine-valine *PRNP* genotypes, suggesting that the BSE agent may infect individuals of all codon 129 genotypes (59). These findings led to an estimated global prevalence of abnormal PrP of up to 1 in 2000 of the UK population (95% confidence interval 1/3500–1/1250) (59). Whether this represents the true prevalence of vCJD infection is still a matter of debate, particularly since the prevalence estimates are not consistent with the small observed numbers of clinical vCJD cases. One interpretation is that there are subclinically infected individuals in the population who may never develop vCJD themselves, but who may be a source of iatrogenic transmission of infection e.g. by blood or organ donation. In light of these unresolved concerns, it is likely that vCJD will remain a public health issue for the foreseeable future.

Pathogenesis of C-BSE

Experimental oral challenges of cattle with C-BSE have been used to study disease pathogenesis in the natural host. In cattle receiving a range of oral doses, transmission was observed in all dose groups, including one of fifteen animals given the lowest dose of 1mg of BSE-infected cattle brain, demonstrating the sensitivity of cattle to C-BSE (76). In experiments where groups of cattle were culled at different intervals following oral infection with BSE, infectivity was first detected in the distal ileal Peyer's patch (PP), and later in the central nervous system and specific sensory ganglia a few months before the onset of clinical disease (51, 68, 135). The distribution of infectivity and PrP^{Sc} in the CNS in preclinical animals suggests that neuroinvasion from the gastrointestinal tract occurs via the autonomic innervation (68, 123). Studies to date have failed to find evidence of infectivity in lymphoid tissues other than the ileal PP and tonsils of preclinical or clinically affected cattle, or in blood and milk (51, 135, 137). At late stages of the disease, there is growing evidence for centrifugal spread of infection along neuronal pathways to tissues such as the tongue and nasal mucosa (13).

Information from these experiments on the distribution of the C-BSE agent in the tissues of infected cattle underpinned the development of a list of tissues defined as Specified Risk Material (SRM). Legislation to enforce the systematic retrieval of SRM from all slaughtered cattle was introduced in the UK (1996) and EU (2001), preventing entry into the human food

chain of significant amounts of C-BSE infectivity (Figure 2). This measure was decisive in limiting the occurrence of human dietary exposure to the C-BSE agent (114).

Experimental modelling of the human transmission barrier to BSE

Careful surveillance and epidemiological studies were instrumental in the identification of zoonotic transmission of classical BSE. However, the success of this approach depended on the occurrence of a large BSE epidemic in the UK cattle population followed by the emergence of a new human prion disease phenotype (variant CJD) in the same country (139). Given the low total numbers of vCJD cases, if transmission of BSE had resulted in a disease similar or indistinguishable from sporadic CJD, it is unlikely that surveillance for human prion diseases in the UK alone would have been able identify its zoonotic properties. Moreover, epidemiology necessarily relies upon the retrospective analysis of events, and therefore obviously offers no possibility of preventing human transmission before it occurs.

In that context, a number of *in vivo* and *in vitro* experimental models have been developed, using C-BSE/vCJD as a benchmark, with the aim of assessing the relative ability of animal prion agents to cross the human transmission barrier (21, 37, 112, 113). Among these models, transmission in non-human primates and transgenic mice expressing human PrP (Table 2) are currently considered as the most informative and reliable approaches (114).

BSE transmits efficiently to cynomolgous macaques by intracerebral and intravenous routes (including blood transfusion), producing similar clinical and neuropathological features to those observed in human patients affected by vCJD (65, 85, 87). The relative efficacy of C-BSE transmission observed in macaques exposed by the oral route to low infectious doses of C-BSE also fits well with the limited number of vCJD cases that occurred in dietary exposed human populations (84). BSE has also been successfully transmitted to other non-human primates including marmosets, squirrel monkeys and lemurs (Table 2) (12, 26). The degree to which different non-human primate species are evolutionarily related to humans may influence the interpretation of zoonotic risk following experimental transmission of animal prions. Since Old World monkeys (including macaques) are more closely related to humans than New World monkeys (e.g. squirrel monkeys) and lemurs, cynomolgus macaques are regarded as a better model to assess the permeability of the human transmission barrier to C-BSE and other animal prion diseases.

The major drawback of using non-human primates in prion research are ethical issues and the long incubation periods following infection, which make such experiments very expensive and severely limit their use in characterizing the zoonotic potential of animal prions.

Following the demonstration that the transmission barrier preventing infection of mice with hamster-adapted scrapie could be removed by expression of hamster PrP^C in transgenic mice (117), several transgenic mouse lines expressing human PrP^C (TgHu) have been developed. These include lines that over-express PrP (30, 118, 126, 134) and gene-targeted lines that express PrP at physiological levels under the control of mouse *PRNP* regulatory elements (24). These mouse models express different variants of the human *PRNP* gene including the methionine/valine (M/V) di-morphism at codon 129, which is a major determinant of susceptibility to human prion disease (92, 143). Most TgHu mouse lines have been shown to propagate human prion diseases, e.g. sCJD, without an apparent species barrier (10, 24, 66, 104).

Transmission of C-BSE to transgenic mice over-expressing human 129MM, 129VV or 129 MV PrP resulted in low attack rates and long incubation times in 129MM and apparent absence of transmission of the agent in 129MV and VV PrP expressing mice (Table 2) (10, 66, 104). Iterative passages of C-BSE into 129 MM mice led to an increase in the attack rate and a slight reduction of the incubation period, but did not result in disease transmission to 129MV- and 129VV-expressing mice (36, 54, 69). In contrast, vCJD isolates transmitted to all three humanized lines, producing a gradation in transmission efficiency from MM (most efficient) to VV (less efficient) (54). In gene-targeted 129MM, 129MV and 129VV mouse lines, inoculation with BSE failed to transmit the infection, while vCJD transmitted to all three lines with a similar gradation in efficiency to that seen in over-expressing transgenic models (24).

Taken together, the results of experimental transmission of C-BSE to TgHu mice converge to indicate a high transmission barrier to C-BSE in humans, and a higher susceptibility of 129MM individuals to infection, which is consistent with the vCJD epidemiological features observed in exposed human populations. The relative efficiency of transmission of C-BSE, vCJD and sCJD to TgHu mice strongly supports the view that these models provide good predictive value of the capacity of prion agents to cross the human transmission barrier. However, these models also have their limitations, including the short lifespan (2-3 years) of mice in comparison to the

long incubation periods (several decades) reported in patients accidentally exposed to human prions (like sCJD contaminated growth hormones) (28).

Scrapie in small ruminants

Classical scrapie is the archetype of prion disease. It was reported for the first time in sheep in the United Kingdom in 1732 and few years later (1759) in Germany. Over the following centuries, scrapie spread to many countries in the world through the export of living animals, and is still endemic in most of these regions (47, 48).
In 1998, a new form of sheep prion disease (named Nor98) was identified in Norway. The epidemiological features and biochemical properties of PrP^{Sc} associated with Nor98 cases clearly differed from scrapie cases that had been previously reported (termed "classical" scrapie), and the disease was therefore considered to be an "atypical" form of scrapie (16).

Classical scrapie can be caused by several prion agent strains. Historically, identification of scrapie strains has relied on characteristic biological phenotypes (incubation period and lesion profile) observed following transmission to a panel of inbred mouse lines. However, the propagation of natural prion isolates into inbred mice lines requires passage through a transmission barrier, which can result in the non-propagation of certain isolates and/or a radical evolution (mutation) of the prion agent they contain (31, 55). Therefore, transmission to conventional mouse models is unlikely to provide a comprehensive and reliable picture of the diversity of prion agents in small ruminants. Transgenic mice expressing ovine or caprine *Prnp* genes display a lower transmission barrier to scrapie agents (61, 117). Serial transmission of about 80 scrapie isolates from Europe in such mouse lines has so far permitted identification of at least four phenotypically distinct classes of classical scrapie agents (18, 21, 127-129). In contrast to classical scrapie, no strain variability has been observed among isolates of atypical scrapie (9, 60, 86).

For the most part, identification of scrapie cases has relied on clinical suspicion (passive surveillance). Starting in 2001, active surveillance for prion diseases of small ruminants was implemented in EU countries, and more recently in a number of other countries e.g. USA, Canada. Active surveillance relies on systematic testing of a proportion of the slaughtered or found-dead animals for the detection PrP^{Sc} in the posterior brainstem. These surveillance programs resulted in the identification of atypical scrapie cases in most EU member states, USA

and Canada, and indeed, atypical scrapie has been incidentally detected in many countries across the world, including those considered free of classical scrapie i.e. Australia and New Zealand (16). Data from the EU active surveillance program clearly demonstrated that earlier evaluations based on passive surveillance had significantly underestimated the prevalence of prion diseases in small ruminants and their geographical distribution (53).

A review of the epidemiology of classical and atypical scrapie in EU member states, using active surveillance data collected between 2002 and 2012, was published by the European Food Standards Agency (EFSA) in 2014 (63). Over this period, totals of 4.7 million sheep and 1.4 million goats were tested, and classical and/or atypical scrapie cases were identified in 25 EU countries. The annual crude prevalence of classical scrapie in the EU was equivalent to about 9 cases per 10,000 tested animals, but prevalence estimates differed considerably among different individual member states. For example, in Cyprus the annual crude prevalence of classical scrapie was between 10 and 800 times higher than that observed in other affected countries. For affected flocks, the within-flock prevalence was on average 20 times higher than the apparent prevalence in the general population identified by active surveillance. Over the same period atypical scrapie was detected with an overall prevalence of about 5.8 cases per 10,000 tested animals. In contrast with classical scrapie, atypical scrapie displayed a similar prevalence over time and in different countries (53).

In both sheep and goats, susceptibility to classical scrapie is strongly influenced by polymorphisms of the gene (*Prnp*) encoding for PrP protein. In sheep, the $A_{136}R_{154}R_{171}$ (denoting amino acids encoded at *Prnp* codons 136, 154 and 171) haplotype is associated with a very high resistance to infection in homozygous individuals. Heterozygous ARR individuals also have a reduced susceptibility to infection (80, 91). In goats, the K_{222}, S_{146} and D_{146} polymorphisms of the *Prnp* gene are also associated with a strong resistance to infection by classical scrapie (43, 105). The EU, the USA and several other countries have implemented breeding policies in sheep, with the aim of control/eradication of classical scrapie by increasing the frequency of the ARR allele in affected flocks, and in the general sheep population. Similarly, several countries are now considering the development of *Prnp* genotype selection programmes for the control and eradication of scrapie in goats.

The association between genetic *Prnp* variations and susceptibility to atypical scrapie is totally different from that observed in classical scrapie. Susceptibility to atypical scrapie is linked to

polymorphisms at *Prnp* codon 141 and 154, and strikingly, ARR allele carriers (both homozygous and heterozygous) that are resistant to classical scrapie can develop the disease (9, 97, 98). This difference means that genetic selection for the ARR allele, which has been the basis of successful control programmes for classical scrapie, will not be effective in controlling atypical scrapie at the flock/herd or population level.

According to the EFSA expert group, the eradication and control measures that were implemented at the EU level from 2001 (including selective breeding for scrapie resistance) were very effective in controlling classical scrapie outbreaks at flock/herd level. However, over the studied period, a statistically significant reduction in classical scrapie prevalence/incidence was demonstrated in only six EU countries. These results demonstrate the difficulties in monitoring the epidemiology of animal prion diseases and the effect of control measures at a population level.

Classical scrapie is a prion disease that, under natural conditions, is acquired from the environment and/or other infected animals by the oral route (48). The within-host dissemination of the classical scrapie agent in naturally exposed small ruminants has been carefully characterized by the study of animals born and raised in endemically infected flocks.
These studies have established that infection usually occurs during the first weeks of life. The scrapie agent enters the host animal via gut-associated lymphoid tissues (GALT) e.g. Peyer's patches, before rapid spread to draining mesenteric lymph nodes and later to all secondary lymphoid organs (3, 133). The amount of infectivity and PrP^{Sc} in lymphoid tissues increases with age before reaching a plateau level. *Prnp* genotype appears to influence the extent of scrapie replication in lymphoid tissues, e.g. sheep heterozygous for the ARR allele have much less detectable PrP^{SC} in the lymphoreticular system (70). The scrapie agent disseminates to the CNS (brain and spinal cord) about halfway through the incubation period, apparently via axonal transport through the enteric (autonomic) nervous system (3, 133). In later stages, the agent appears to redistribute (centrifugally) from the central to the peripheral nervous system and skeletal muscle (5). In blood, the infectious agent can be detected as early as three months of age and persists throughout the incubation period (83). In clinically normal ewes infected with scrapie, the placenta accumulates large amounts of infectivity, and plays a major role in the dissemination of the prion into the environment and to other individuals (81, 111, 131). Similarly colostrum and milk were shown to contain infectivity, and their capacity to transmit disease to suckling lambs was demonstrated (78, 82).

The pathogenesis of atypical scrapie has not been characterized to the same extent. Initial investigations failed to identify PrP^{Sc} accumulation in peripheral (non-neuronal) tissues collected from field cases or experimental atypical scrapie cases (4, 16). The apparent restriction of the agent to the CNS was interpreted to support the hypothesis that atypical scrapie could be a spontaneous disorder of PrP folding and metabolism, occurring in aged animals without external cause (16). In addition, there was no statistical difference in the prevalence of atypical scrapie between the general population and flocks where a positive case had been identified, providing further support for the idea that atypical scrapie may not be a contagious disease (52). However, low levels of infectivity have been detected in skeletal muscle, peripheral nerves and lymphoid tissues of animals infected naturally or experimentally with atypical scrapie (4). In the same study, brain samples containing very high levels of infectivity were negative for PrP^{Sc} using the most sensitive current diagnostic tests. Moreover, atypical scrapie can be experimentally transmitted via the oral route in small ruminants, resulting in a similar clinic-pathological phenotype to that observed in natural cases (122). These findings mean that the origin and aetiology of atypical scrapie (spontaneous disorder versus acquired disease) remains an open question.

Zoonotic potential of classical and atypical scrapie

Several epidemiological studies, generally based on case-control approaches, have failed to identify exposure to small ruminants or small ruminant products as a risk factor for developing CJD (29, 132). Countries considered to be scrapie-free, such as Australia and New Zealand, display a similar sCJD prevalence to countries affected with scrapie. In addition, there is no apparent difference in the range of clinical and pathological manifestations of human TSE cases between scrapie affected countries and those designated scrapie free. Together, these different lines of evidence have led to the general conclusion that small ruminant prion diseases were of negligible risk to humans.

Recently cases of atypical scrapie were confirmed in both Australia and New Zealand, casting serious doubt on the validity of one of the most popular arguments used to reject the zoonotic potential of small ruminant prion diseases (42, 73). More generally, active surveillance programs for TSE in small ruminants revealed how inaccurate was knowledge on the prevalence and geographical distribution of TSEs in small ruminants (53), and highlighted the

limited value of past epidemiological studies aiming at assessing the zoonotic potential of animal TSEs.

Data collected through active surveillance programmes offer an opportunity to reassess the zoonotic potential of small ruminants TSEs through informed and modern epidemiological investigations. However, the incubation period for prion disease in humans after exposure to prions *via* peripheral routes, such as in cases of iatrogenic CJD and kuru, can exceed several decades (28, 39). In this context, it will be a challenge to combine epidemiological data collected contemporaneously in animal and human populations to determine the existence of a causative link between prion disease occurrences in these different hosts.

Seminal early experiments failed to demonstrate the transmissibility of classical scrapie in non-human primates (58). However, subsequently one sheep classical scrapie isolate was transmitted to two intra-cerebrally challenged marmosets (Table 2). The incubation periods observed with this scrapie isolate were slightly shorter than with a cattle BSE isolate, suggesting that that transmission barrier for both isolates might not be different (12). More recently, successful transmission of a classical scrapie isolate to a cynomolgus macaque has been described. The incubation period in this animal was prolonged (> 10 years following intracerebral challenge) and the neuropathology observed was unique in comparison to other animal prion diseases (C-BSE, L-BSE) transmitted in this model (41). To date there are no available results concerning the experimental transmission of atypical scrapie to non-human primates (Table 2).

The transmission of both classical and atypical scrapie isolates to TgHu mice has been tested in several studies (Table 2). In gene-targeted transgenic mouse lines expressing physiological levels of human PrP and challenged intracerebrally with a number of natural sheep and goat scrapie isolates, there was no evidence of infection or clinical disease following primary passage (107, 141, 142). However, serial passages in the same transgenic mouse lines, which could allow the identification of subclinical infection, were not performed in these studies. The same mouse lines were also shown to be resistant to cattle BSE (25), and therefore this model does not allow assessment of whether the zoonotic potential of classical or atypical scrapie is lower or higher than that of BSE.

In a more recent study, a panel of classical scrapie isolates was tested in transgenic mouse lines that over-express human PrP (36). The mouse lines represented 129MM (tg340), 129VV

(tg361) and 129MV (F1 cross of tg340 and tg361) *PRNP* genotypes. Following intracerebral inoculation of the three mouse lines with a panel of six natural sheep scrapie isolates (collected in EU countries between 1994 and 2002), clinical disease was not identified in any inoculated mice, but PrP^{Sc} accumulation was observed in the brain of two out of six 129MV mice infected with a single scrapie isolate. Serial passage of the isolates in the same mouse lines led after second passage to positive transmission, resulting in clinical signs in mice inoculated with three of six isolates. Interestingly, the sheep scrapie prions that propagated in humanized transgenic mouse models displayed a transmission efficiency (attack rate on first and second passage) that was comparable to that of cattle BSE. After third passage, the propagated prions displayed a phenotype (incubation periods and PrP^{Sc} distribution in the brain) that was identical to those causing sporadic CJD (sCJD) in humans. This last finding raised important questions about the possible link between TSE in small ruminants and occurrence of human TSE cases. These transmission experiments unambiguously showed that sheep scrapie prions propagate in mice that express variants of human PrP. While the efficiency of transmission at primary passage was low, subsequent passages resulted in a highly virulent prion disease in human PrP expressing mice.

Atypical BSE

The implementation by the EU of an active surveillance system in ruminant livestock in 2001, involving testing of healthy slaughtered cattle and fallen stock (Figure 2), led to the discovery of "atypical" cases of BSE in cattle, which were often not associated with overt neurological abnormalities/disease. Two atypical phenotypes were observed, categorized on the basis of low and high apparent molecular masses of unglycosylated protease-resistant PrP on Western blots, and were termed as bovine amyloidotic spongiform encephalopathy (BASE) or L-BSE and H-BSE, respectively (22, 35).

Transmission of atypical BSE isolates in various mouse models clearly demonstrated that H- and L-type BSE cases are caused by specific prion strains that differ from the classical BSE/vCJD agent (18, 19, 34, 75). The transmission of H-BSE field isolates to transgenic mice expressing bovine PrP resulted in the propagation of an agent with phenotypic characteristics of classical BSE in some animals (130). This suggested that a low level of C-BSE agent could be present in a proportion of the H-BSE isolates and might represent a source for C-BSE re-emergence in cattle

The L-BSE and H-BSE cases reported so far were mainly detected in asymptomatic cattle 8 years of age or older, in contrast to C-BSE, where the majority of cases were 4-6 years old. Since 2001, a total of 60 cases of L-BSE and 44 cases of H-BSE were reported in the whole EU (Table 1). Epidemiological studies in the French cattle population indicated that the apparent prevalence of atypical BSE cases is very low (1.9 cases H-BSE and 1.7 cases L-BSE per million tested cattle over 8 years old) (23). Outside the EU, rare atypical BSE cases have been reported in Japan, the USA, Canada, Switzerland and Brazil (50, 88, 115).

The origin of H-BSE and L-BSE cases is unknown. It has been argued that the low prevalence and the advanced age of positive animals provide evidence for a spontaneous origin of atypical BSE, but an infectious etiology cannot be definitively ruled out.

Limited tissues are available from L-BSE and H-BSE cases identified through surveillance, therefore experimental transmission studies in cattle are the main source of information concerning the pathogenesis of atypical BSE agents. Following IC challenge, abnormal prion protein accumulation was identified in the central nervous system (brain, spinal cord and retina), the peripheral nervous system (autonomic and motor) and at lower levels in skeletal muscle (muscle spindles) of affected animals. No consistent prion accumulation was detected in the lymphoid organs (13, 77, 100, 124). Following oral challenge of cattle with a range of doses of L-BSE infected cattle brain homogenate, only one animal that received the highest dose (50g) developed neurological clinical signs. This animal showed a similar tissue distribution of PrP^{Sc} to that observed in IC challenged animals (central & peripheral nervous system), although there were subtle differences in the distribution of PrP^{Sc} within the brain regions (101).

Other experiments in which cattle were orally challenged with L-BSE and H-BSE are still ongoing, and the final analysis of the pathogenesis of atypical BSE in comparison to C-BSE awaits their results. However, the examination of a limited panel of tissues collected from natural L-BSE cases in Italy confirmed the apparent restriction of the prion to the central and peripheral nervous system (124).

Due to the low apparent prevalence of atypical BSE and the design of cattle TSE surveillance programs, it is unlikely that atypical BSE cases are efficiently detected. Therefore, it should be

assumed that small numbers of atypical BSE incubating cattle are entering the human food chain. The many uncertainties related to the distribution of atypical BSE cases in the cattle population mean that epidemiological approaches are unlikely to be informative in assessing the zoonotic abilities of these prions.

Intracerebral injection of L-BSE isolates from European and Japanese cattle into cynomolgus macaques resulted in disease transmission with shorter survival times than in C-BSE infected macaques (23–25 months for L-BSE versus 38–40 months C-BSE) (Table 2) (40, 103). In contrast, no positive transmission was reported in macaques that were inoculated with H type BSE (41). Inoculation of L-BSE, H-BSE and C-BSE isolates into transgenic mice over-expressing human PrP demonstrated that L-BSE transmitted more efficiently than C-BSE on primary transmission, with 100% attack rates and no shortening of incubation periods on subsequent sub-passage (Table 2). In contrast, H-BSE isolates failed to transmit in the same mouse model (20). Another study using a different transgenic mouse model expressing human 129M PrP also showed efficient primary transmission of L-BSE (75). Biological strain typing of L-BSE and a panel of sCJD subtypes in human PrP transgenic mice failed to find any evidence that L-BSE causes a recognised form of sCJD (69).

Collectively, these results support the view that H-BSE agent has a low zoonotic potential. In contrast, L-BSE displays an equal or greater virulence than C-BSE in primate and TgHu mouse models, and may therefore pose a higher risk of zoonotic infection.

Chronic Wasting Disease

Chronic wasting disease (CWD) was first recognized as a disease of captive mule deer in Colorado (USA) during the 1960s, and confirmed to be a prion disease in 1980 (140). Since then the CWD affected area of North America has considerably expanded, with the disease now having been identified in 20 US states and two Canadian provinces (Saskatchewan and Alberta). CWD has become endemic in wild and captive cervid populations in most of these regions. It affects the majority of North American endemic cervid species, with the notable exception of free-ranging caribou in Canada. The prevalence of CWD in free-ranging cervids varies across North America, but can be as high as 30% in some areas, and the spread of CWD in North America is likely to be irreversible (140). Outside North America, CWD infection has been confirmed in captive cervids in South Korea as a result of importation of sub-clinically infected

animals from Canada (72). Despite the implementation of vigorous control and eradication measures CWD is still present in South Korea.

In April 2016, CWD was found in a free-ranging reindeer (*Rangifer tarandus)* population in the Nordfjella region of Norway, and then later (June 2016) in two European moose (*Alces alces*), in a different area of the country (17). This led to intensive active surveillance across Norway, and the implementation of a culling programme in the affected wild reindeer population. At the time of writing, 17 additional cases of CWD have been identified in reindeer from Nordfjella. One additional moose and one red deer from Norway also tested positive, and a further CWD-infected moose was identified in Finland. At this stage it is still unclear whether the discovery of CWD in Europe has any relationship with the epidemic observed in North America. Epidemiological studies based on large scale testing of free-ranging cervids were recently implemented by several northern European countries, and should be helpful in documenting the geographical distribution and prevalence of CWD in European cervid populations.

There is clear evidence demonstrating that several prion strains are responsible for CWD in North American cervids. Inoculation of a panel of CWD isolates from various species and geographic locations in North America into transgenic mice over-expressing cervid PrP indicated the presence of at least two CWD prion strains (referred to as CWD1 and CWD2) that circulate either independently or as a strain mixture (7). Bioassays in heterologous PrP transgenic mouse models or in conventional rodent models are consistent with these results (44, 125). However, it is unlikely that the strain typing work carried out so far has provided a definitive picture of the diversity of CWD strains that are circulating in North American cervid populations. Transmission experiments for strain typing of Norwegian CWD isolates are still ongoing, and thus definitive results are not yet available. Preliminary data presented by several teams in the 2018 International Prion Congress suggests that the phenotypic characteristics of the Norwegian isolates are different from those observed so far in North American isolates.

In North America, CWD pathogenesis has been investigated using both naturally exposed and experimentally challenged animals. Under natural exposure, the infection apparently occurs by the oral route following contact with an infected individual or contaminated environment (96). The pathogenesis and PrP^{Sc} distribution in CWD are very similar to that reported in classical scrapie in small ruminants. Initial entry of the agent occurs through tonsils and GALT with

rapid involvement of the lymphoreticular system (LRS) and the enteric nervous system, followed by neuroinvasion of the CNS via autonomic nervous system pathways (56, 110, 119, 121). Involvement of the LRS seems to vary between deer and wapiti, with less abnormal PrP deposition in the lymphoid tissues of wapiti compared with deer (110). PrP^{Sc} has been detected in a large number tissues of affected deer, including those commonly consumed as venison (heart, skeletal muscles, tongue, liver, kidneys) or used as 'natural medicine' (antler velvet)(6, 8, 94, 120).

In both captive and wild cervids, CWD has been demonstrated to be highly contagious (93). During the preclinical phase of infection, the CWD agent has been demonstrated in placenta, saliva, faeces and urine, which are all likely to contribute both to inter-individual transmission and contamination of the environment (62, 89).

So far, 16 amino acid polymorphisms have been described in the PrP sequence of different cervid species, some of which are associated with lower rates of CWD infection and slower progression of the disease in natural hosts (116). For instance, in wapiti (*Cervus canadensis nelsoni*) the L132 allele (versus M132) is associated with partial protection against CWD infection (99), while in mule deer (*Odocoileus hemionus*) the S/F dimorphism at codon 225 influences susceptibility to the disease, the 225F allele being partially protective against infection under natural exposure conditions (71). As in small ruminants affected with classical scrapie, the selection of PrP CWD resistant alleles could be an effective means to control the disease and prevent cervid depopulation in endemic areas, at least in captive herds. However, none of the polymorphisms identified so far seems to provide a sufficient level of disease protection to make genetic selection a feasible proposition for control of CWD.

No new or unusual form of human TSE has been identified so far in countries where CWD epidemics have developed (2). Comparison of the CJD prevalence rates between North America and other countries (Europe) does not suggest that CWD is responsible for a detectable increase in human prion disease prevalence. A retrospective study carried out using State death registry data collected between 1979 and 2001 in Colorado failed to identify any statistical difference in CJD prevalence between CWD endemic versus CWD non-endemic counties (90). In the US, retrospective investigations identified several sCJD cases who had a history of potential or demonstrated exposure to CWD prions through venison consumption or hunting cervids in affected areas (15), but longitudinal studies related to known dietary exposure to CWD failed

to demonstrate an increased risk of developing a prion disease in these individuals (102). Nevertheless, considering the limited duration of the observation period in these studies (in comparison with the potentially very long incubation periods for TSE in man), the significance of these observations remain uncertain. Collectively the epidemiological data suggest a lack of causative link between CWD epidemics in North America and the occurrence of human TSEs. However, because of the considerable limitations of the data supporting this statement, it would probably be unwise at this stage to conclude an absence of zoonotic risk associated with CWD agents.

Although CWD was relatively recently identified, a number of studies specifically aiming at clarifying the capacity of CWD agent(s) to cross the human species barrier have already been carried out through experimental inoculation of TgHu mice and primates. A total of seven CWD isolates have been inoculated into different conventional (wild-type) and TgHu mouse models, including models that expressed the 129M and/or the 129V variants of the *PRNP* gene (Table 2). The lack of clinical disease or PrP^{Sc} accumulation in the brains of the inoculated mice indicates the existence of a substantial transmission barrier (74, 79, 125, 142). However, it should be noted that these studies did not include secondary passage in TgHu mice, which was necessary for revealing the transmissibility of scrapie isolates and certain C-BSE isolates in similar models (36).

Two separate studies have attempted to transmit CWD isolates to cynomolgus macaques (Table 2). In the first study, cynomolgus macaques and squirrel monkeys were challenged by intracerebral and oral routes with CWD-infected brain homogenate. Although squirrel monkeys proved to be susceptible to CWD, there has been no evidence of transmission in macaques after observation periods ranging from 1.5 to over 13 years post inoculation (109) . In the second study, eighteen cynomolgus macaques were challenged with CWD by different routes, including oral inoculation with muscle tissue from CWD-infected deer. The results of this study are still unpublished, but presentations at recent international scientific conferences and meetings (https://www.cdc.gov/prions/cwd/transmission.html) described the occurrence of neurodegenerative disease 4.5 to 6.3 years post inoculation in 5 out of 18 of the cynomolgus macaques that had been exposed by either the intracerebral (n=2) or the oral route (n=3). Faint but consistent PrP^{Sc} accumulation and amyloid seeding activity were observed in the central nervous system of clinically affected animals, which strongly supports the view that the propagation of a prion disease was the cause of the neurodegenerative disorder. The reasons for

the discrepancy between the results from these two macaque transmission experiments are not clear, but may relate to differences in the oral dosing regimen and/or differences in CWD strains present in the inocula used. A more definitive analysis awaits completion and publication of the second study, but these preliminary data have prompted renewed concern about the potential for transmission of CWD to humans.

Conclusions:

Thirty years ago the BSE crisis brought the previously obscure 'prion diseases' into the world spotlight. Since then, our comprehension of the properties and biology of prions has progressed remarkably. However, despite the massive efforts that were deployed, there are still major unanswered questions about animal prion diseases and the risk they might represent for the human population.

The recent discovery of atypical forms of BSE and scrapie, the identification of CWD cases in Europe, and the recognition of a novel prion disease of camels within the past year, illustrate beyond any words the limits of our knowledge in this field.

There is no epidemiological evidence that classical or atypical scrapie, atypical forms of BSE, or chronic wasting disease (CWD) are associated with human prion disease but the limitations of the epidemiological data should be taken into account when interpreting these results. Transmission experiments in non-human primates and human PrP transgenic mice clearly illustrate that classical scrapie, L-type atypical BSE (L-BSE) and CWD may have zoonotic potential. However, it is still difficult to predict from these results the likelihood that an animal prion disease will transmit to humans under conditions of field exposure.

The profound uncertainties we are still facing concerning the zoonotic abilities of prion diseases of livestock emphasise the importance of maintaining the effective but expensive measures (SRM retrieval, surveillance program, feed ban on animal proteins) that were implemented during the BSE crisis (Figure 2) to prevent human exposure to these pathogens.

Figure Legends

Figure 1: Number of confirmed C-BSE cases (Logarithmic scale) reported to the OIE (Office International des Epizooties) by the UK, the European Union (excluding the UK) and the rest of the world, by year.

Implementation dates of key control measures (bans on the use of meat and bone meal in farm animal feedstuffs) and active surveillance system (post mortem testing of slaughtered cattle and fallen stock) in the UK and the EU (also applicable to UK) are indicated on the graph.

Figure 2: Timeline of major events and control measures for protection of animal and human health during the C-BSE epidemics in the UK and European Union.

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Table1: Number of cattle tested post mortem and numbers of confirmed classical and atypical BSE cases in the European Union per year.

	2001	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**
Total number of tested cattle (in millions)	8.516	10.423	10.041	11.049	10.113	10.047	9.692	10.051	7.467	7.504	6.361	4.795	3.135	2.287	1.423	1.352
C-BSE cases	2174	2129	1334	848	542	323	157	117	55	37	23	12	3	3	3	1
H-BSE cases	2	3	4	2	3	3	5	5	6	4	4	1	4	2	2	4
L-BSE cases	0	5	4	4	4	4	8	6	5	4	3	6	1	6	1	0

Table 2 : Transmissibility of animal prion diseases in animal models of the human species barrier

Prion diseases		Positive transmission reported in animal models of the human species barrier		
		New World Primates†	**Old world Primates††**	**Human PrP expressing transgenic mice***
Cattle	C-BSE	Yes (IC and oral route) (12)	Yes (IC, intravenous and oral route) (65, 84, 87)	Yes (10, 36, 54,66, 69, 104) No (25)
	Atypical H-BSE	-	still ongoing (IC route) (41)	No (20, 142)
	Atypical L-BSE	-	Yes (IC route) (40, 103)	Yes (20, 69, 75) No (142)
Small Ruminants	Classical Scrapie**	Yes (IC route) (12)	Yes (IC Route) (41)	Yes (36)
	Atypical Scrapie	-	Ongoing experiment (IC route) (41)	No (107, 141, 142)
Cervids	CWD** (North American isolates)	Yes (IC and oral route) (109)	No (IC and oral route) (109) Yes (IC and oral route) ‡	No (74, 125, 142)

†: Squirrel monkeys or Marmoset
††: Cynomolgus Macaques –
*: Mice were all inoculated by the intracerebral route (IC) –
**: These prion diseases are associated with multiple prion strains
‡ Czub *et al.* personal communication: https://www.cdc.gov/prions/cwd/transmission.html

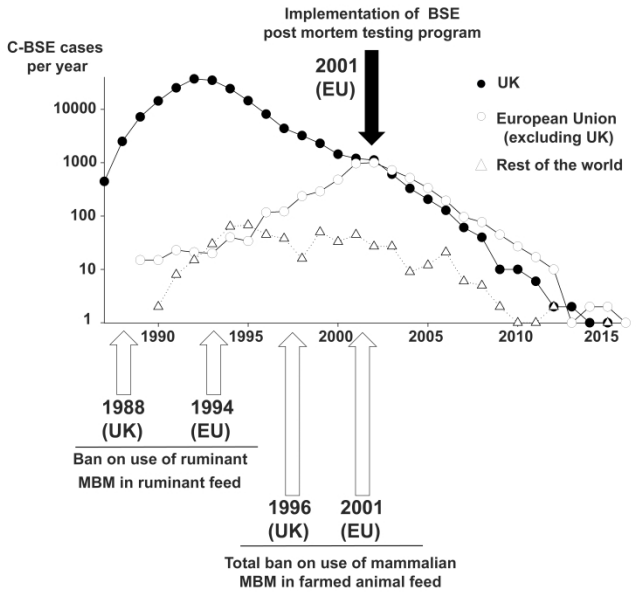

Figure 1 : Number of confirmed C-BSE cases (Logarithmic scale) reported to the OIE (Office International des Epizooties) by the UK, the European union (Excluding the UK) and the rest of the world by year. Implementation dates of key control measures (bans on the use of meat and bone meal in farm animal feedstuffs) and active surveillance system (post mortem testing of slaughtered cattle and fallen stock) in the UK and the EU (also applicable to UK) are indicated on the graph.

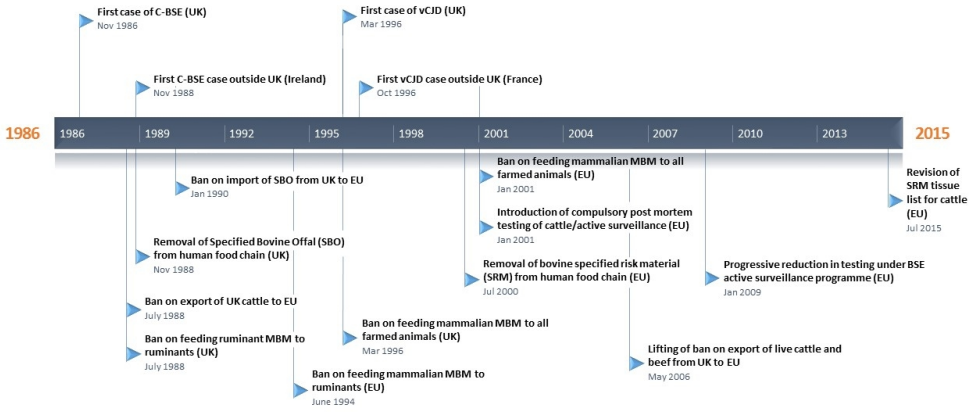

Figure 2: Timeline of major events and control measures for protection of animal and human health during the C-BSE epidemics in the UK and EU.

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