Genetics of Prion Disease in Cattle

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ABSTRACT: Bovine spongiform encephalopathy (BSE) is a prion disease that is invariably fatal in cattle and has been implicated as a significant human health risk. As a transmissible disease of livestock, it has impacted food safety, production practices, global trade, and profitability. Genetic polymorphisms that alter the prion protein in humans and sheep are associated with transmissible spongiform encephalopathy susceptibility or resistance. In contrast, there is no strong evidence that nonsynonymous mutations in the bovine prion gene (*PRNP*) are associated with classical BSE (C-BSE) disease susceptibility, though two bovine *PRNP* insertion/deletion polymorphisms, in the putative region, are associated with susceptibility to C-BSE. However, these associations do not explain the full extent of BSE susceptibility, and loci outside of *PRNP* appear to be associated with disease incidence in some cattle populations. This article provides a review of the current state of genetic knowledge regarding prion diseases in cattle.

KEYWORDS: prion, BSE, cattle, PRNP

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Background

Prion diseases, referred to as transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative diseases that affect humans as well as other mammals (reviewed by Prusiner).[1] Prion diseases are mediated and transmitted by an infectious misfolded protein.[2] This class of diseases present with a spectrum of neuropathologically mediated symptoms, manifested by the fact that the central nervous system (CNS) is the primary target tissue and is where the etiology of disease is noted.[3] The common TSE pathologies include spongiform changes, neuronal loss, glial cell activation, and most notably the accumulation of amyloid aggregates.[4] Pathological manifestations are progressive, cumulative, and inevitably fatal given sufficient time. In humans, several neurological diseases have been identified and are currently regarded as prion diseases, including Creutzfeldt-Jakob disease (CJD), Kuru, fatal familial insomnia, and Gerstmann-Sträussler-Scheinker syndrome.[1]

Prion disease in ruminants was documented as far back as the 18th century, specifically scrapie in sheep, and has been documented in the United States for some time (since ~1947). However, the recent effort of the National Scrapie Eradication Program, initiated by the U.S. Department of Agriculture has effectively reduced the prevalence of prion disease in U.S. sheep (PrP^{Sc}) by over 85% (http://www.eradicatescrapie.org). In North America, the current most prevalent prion disease is in wildlife, chronic wasting disease (CWD), which has persistently affected deer, elk, and moose populations. Arguably, the most famous prion disease in ruminant is bovine spongiform encephalopathy (BSE) in cattle sometimes referred to as *mad cow* disease by popular press. This term was coined as a result of the U.K. BSE Epidemic in the late 1980s to the early 1990s and the abnormal behavior observed in afflicted cattle. This epidemic recorded approximately 170,000 verified infected cattle and the massive slaughter of ~4.4 million cattle to curtail the potential transmission of disease to humans, called variant CJD (vCJD). This animal culling was obviously at a substantial economic cost, and it is well accepted that BSE has cost the global beef market hundreds of billions of dollars in lost sales revenues and testing and mitigation costs. Interestingly, a lone case of BSE in Washington state in December 2003 resulted in roughly 3.5 billion dollars in reduced export of beef in 2004 (http://www.bookstore.ksre.ksu.edu/pubs/MF2678.pdf).

There are three known manners by which prion diseases can develop. Prion diseases can manifest through acquired transmission, they can develop in accordance with inherited genetic risk, or through sporadic origins. Acquired prion diseases in cattle are transmitted primarily by oral exposure to infectious misfolded prions, but in humans this may also include routes such as contaminated human products (ie, corneal graft, growth hormone, or gonadotropin) or

surgical instruments and is known as iatrogenic transmission.[5] With genetic risk, rather than direct exposure to exogenous misfolded prion protein, there exists a genetic and cognate protein sequence variation that increases the probability that the native prion protein will spontaneously misfold to *seed* the disease. Sporadic refers to all other cases where the exact exposure to the misfolded prion protein is unknown but is not believed to be acquired or genetic. Importantly, all prion diseases involve the modification of the native prion protein PrP^{C} into a misfolded infectious form. This misfolding cascade event is described by Watts et al.[6] as "epigenetic templated protein misfolding", although the process by which one misfolded infectious prion protein initiates an alteration in the folding of an existing native prion protein remains unclear. Regardless, the hallmark feature of the misfolded protein is an induced change in its secondary structure, from a predominantly α-helices–containing protein to one with an increase in the β-pleated sheet content.[7] The increase in the β-pleated sheet content of the protein greatly enhances its protease resistance to digestion with proteinase K and hence the nomenclature PrP^{Res} where the Res superscript refers to the resistance to proteinase K–mediated degradation.[8] This resistance to normal protein degradation is in part responsible for the accumulation of the abnormally folded cellular protein that aggregates as an intracellular plaque. However, not all PrP^{Res} disease–causing molecules are the same; rather, they consist of distinct strains with different modes of transmission and varied biochemical and neuropathological properties.

The past few decades have spawned much research interest and effort toward understanding prion diseases, their etiology, transmissibility, and the causation of "the expanding universe of prion diseases".[6] The breadth of prion research has recently declined; primarily due to reduced funding priorities as the incidence of BSE and its potential impact on human health has been reduced by improved management policies. The first such policy was to ban feeding ruminant-derived meat and bone meal to ruminants, which was later extended to all farm animals, "the real feed ban" (http://www.cdc.gov/prions/bse/feed-ban.html). The second management policy specifically addresses how the tissues with the potential of harboring accumulated infectious prion particles, termed *specified risk material* (SRM), are disposed of as to prevent this material from ever entering the food chain. However, still of research interest and direct relevance to human health is the concept that an alternative conformational state of a protein can change, and thereby "infect", other proteins by changing their cognate conformation. This conceptualization of a new type of pathogenic material has had a profound influence on a large number of human neurodegenerative diseases, including Alzheimer's and Parkinson's disease. It is hoped that this brief review, although with a primary focus on cattle, will effectively summarize some of what is generally known about prion diseases and stimulate future avenues of TSE research and perhaps even generate renewed interests within the scientific community.

Etiology and Manifestation of Disease

In classical prion diseases of humans, following the initial onset of loss of memory, altered behavior, and communication problems, there is rapidly progressive dementia accompanied by the manifestation of imbalance and ataxia. Similarly in cattle, clinical signs include progressive neurological and behavioral changes (increased nervousness, apprehension, and aggressiveness), altered gait or movement (tremors, weakness, and hind limb ataxia), and weight loss.[9–11] Other disease characteristics include neuropathological changes within the CNS and include the presence of distinct vacuoles known as spongiform changes, neuronal loss, glial activation, and accumulation of intracellular neuronal amyloid aggregates due to misfolded host prion protein.[12] To add to the complexity of the disease etiology and progression, prion diseases have long, variable, and somewhat unpredictable incubation periods. For instance, a potentially conservative estimate of the typical incubation time for onset of BSE in cattle ranges from 2 to 8 years (http://www.cfsph.iastate.edu/Factsheets/pdfs/bovine_spongiform_encephalopathy.pdf). Furthermore, it is possible that an animal is perceived as *resistant* to disease because it does not exhibit clinical signs and does not succumb to the pathologies associated with prion disease within their normal production life span. Although it is hypothesized that there is a genetic component that contributes to incubation time, the dose of misfolded prion exposure has the largest effect on disease progression, with larger infectious doses resulting in more rapid development of disease, which is not unlike other known pathogen-mediated diseases.

It stands to reason that there is great interest in the capacity to identify afflicted animals quickly, so as to reduce their potential to spread the disease. While we may recognize the onset of clinical signs of prion diseases, currently there is neither treatment nor a definitive antemortem diagnostic method. To date, definitive diagnoses of prion diseases in cattle require postmortem examination of brain tissue. Alternative protocols with sufficient sensitivity and accuracy for live animal tests have yet to be effectively developed. Prion disease in cattle is initially diagnosed via ELISA (enzyme-linked immunosorbent assay). This test quantitatively detects the presence of misfolded prion protein, but this test can yield inconclusive results. Animals are diagnostically confirmed for prion disease by immunohistochemical detection of prion protein accumulation, detection of neuropathological features such as vacuoles, or western blot analysis (http://www.bseinfo.org/bsediagnosis.aspx). A western blot analysis, also referred to as protein immunoblot, is an important technique employed in prion disease by researchers as it has the advantage of being able to determine protein size through electrophoresis separation, unlike ELISA. Thus, western blot analysis is the method used

to characterize and differentiate the various types or strains of prion disease,[13,14] to be described later in this review.

Function and Organization and of Native Prion Protein

The prion protein (PrP) is a polypeptide which, prior to any posttranslational modifications, is approximately 264 amino acids long in cattle, but varies slightly between species. Many cellular proteins are modified posttranslationally, and the prion protein has two glycosylation sites. Western blot analyses have revealed that the cognate prion protein is routinely present in a mixed state of mono-, di-, and unglycosylated banding patterns in humans, mice, and cattle. This plasma membrane glycoprotein, in general, has two domains, a flexible and unstructured or disordered N-terminal domain and an ordered, stable C-terminal domain. The N-terminal domain contains an octapeptide-repeat region that has the ability to bind copper.[4] This region of the mature protein is cleaved in the endoplasmic reticulum and further modified to include a glycosyl-phosphatidyl-inositol (GPI) anchor. The GPI anchor allows the prion protein to be targeted and attached to the exterior of cholesterol and sphingolipid-rich membrane subdomains termed *lipid rafts*. These rafts are detergent-resistant subdomains of the plasma membrane phospholipid bilayer. The mature PrP^C undergoes a cycle where it is endocytosed and either shuttles back to the cell surface in the lipid rafts or is eventually targeted for degradation in lysosomes.[15] So the normal prion protein can be classified as a GPI-anchored extracellular protein. Expression of the native protein PrP^C is abundant in the brain, digestive tract, and lymphoid tissues and also the kidney, skin, mammary gland, muscle, and heart. As it turns out, the prion protein is generally well conserved among species with both a similar amino acid sequence and secondary protein structure. It is often the case that essential proteins show higher levels of conservation across species than less essential proteins or ones that are components of more redundant biological processes or pathways. Therefore, it was initially speculated that the native prion protein must play some essential biological role. However, when *Prnp* knockout mice were shown to lack any dramatic phenotypic effect as compared to wild-type mice, the high degree of conservation of *Prnp* is a bit more *evolutionarily perplexing*.[16] As mentioned, GPI-anchored proteins are known to participate in lipid raft formation with the plasma membrane, influence aggregation of extracellular cell signal receptors as well as major histocompatibility factors (MHC's) for recognition of *self*, and serve as docking sites for viruses. PrP^C is thought to be involved in many critical biological processes including cell signaling, proliferation, survival, differentiation, and apoptosis[17] (for reviews, see Chen et al, Westergard et al, and Kurschner et al).[18–20] The lack of pronounced phenotype associated with loss of the native prion protein may be a reflection of a redundancy built into GPI-anchored protein–mediated cellular events. It should be noted that many studies demonstrate a protective role of PrP^C against oxidative damage, conferring redox properties to PrP^C (reviewed in Linden et al).[21] Although researchers continue to investigate the biological role of PrP^C,[22,23] it is well established that the presence and overall abundance of this protein is essential for the manifestation and transmission of prion disease.

Prion Disease Transmission

As mentioned earlier, the source, causation, or acquisition of prion diseases can be through exposure to infectious sources of misfolded prion protein, inherited genetically, or sporadic occurrence in nature. To date, there are ~190,660 cases of BSE reported in 28 countries worldwide (http://www.oie.int/animal-health-in-the-world/bse-specific-data). The most common prion disease in cattle, termed classical BSE (C-BSE), is typically acquired through the consumption of meat and bone meal contaminated with the infectious prion agent. While there are several different theories as to how C-BSE originated in cattle, no one theory has been accepted by the scientific community. In addition to cattle, other mammals including humans have been known to contract prion diseases from oral ingestion. One specific example is Kuru in Fore population of Papua New Guinea who contracted prion disease through the practice of ritualistic cannibalism of their deceased. An additional but important example of cross-species transmission is vCJD, a human prion disease suspected to be caused by dietary exposure to products from BSE-infected cattle[24–27] and a major reason for the extensive economic cost of BSE.

Less common types of prion disease in cattle are collectively termed atypical BSE. Atypical BSE diseases may have manifested by genetic (spontaneous) and/or sporadic routes, and unlike C-BSE, these are not thought to be acquired from oral ingestion of contaminated feed. The genetic development of prion disease due to nucleotide mutations within the gene that codes for the prion protein can be subsequently inherited through the germ line. One recorded case of protein-coding mutation associated with disease in cattle is at codon 211 (E211K),[28,29] synonymous to the E200K mutation found in some cases of CJD.[30] Prion diseases that have not been inherited or transmitted through other known infectious sources are generally termed sporadic, and in general, the scientific community does not have a good understanding as to the cause of sporadic prion diseases. However, regardless of the origin of the prion disease, tissues in cattle known to be able to accumulate infectious prions (brain, spinal cord, intestinal tract, and tonsils) are termed *specified risk material* (SRM) and have strict disposal protocols to prevent these tissues from entering the food chain.

It is important to emphasize that while a hypothesis for proposed routes of prion migration from oral introduction to CNS disease manifestations has been suggested,[3] the exact nature of this transmission remains presumptive. In cattle, however, based on the identification of the initial location of accumulated misfolded prion protein, the primary

site of entry and thus infectivity is the ileal Peyer's patches.[31] In some TSE, there is potential for *horizontal transmission*, which simply means transmission directly from one animal to an adjacent animal in the herd. However, unlike scrapie in sheep and CWD in deer where horizontal transmission has been shown,[32–35] there is no evidence of horizontal transmission of BSE in cattle. Another proposed route of disease exposure is termed *vertical transmission*, which is best explained as transmission mainly from parents to offspring either *in utero* or through birth or lactation. While the possibility of vertical transmission has not been entirely excluded in cattle, it is considered to be very low in incidence if at all, and there is no evidence of prion transmission in milk by ELISA detection,[36] embryos, or semen[37] via histopathology, immunohistochemistry, or bioassay detection.

Prion Disease Strains

Prion strains variation is not necessarily dependent on genetic variation in the prion gene. Rather, in prion disease, a strain and strain typing are primarily based upon biochemical differences, the degree of N-glycosylation, resistance to proteinase K, as well as the pathological profile and susceptibility to infect other species. Western blot analysis is used to identify variation of the prion protein by identifying the relative amounts and molecular weights of di, mono, and unglycosylated types. Histopathology is used to determine presence and specific location of vacuoles and immunohistochemistry, using labeled antibody-specific binding, and enables visualization of prion accumulation. These combined techniques provide useful information regarding the presence and location of vacuoles, the type of aggregate formed, and the location of these events which are used to characterize different strains. Further, these techniques have been utilized for most of the known original isolates of infectious prions and have generated our current understanding of prion strain types. In humans, CJD strain types are known to differ depending on the amino acid (methionine or valine) at codon 129. More specifically, this single-nucleotide polymorphism (SNP) rs1799990 at codon 129 of the *PRNP* gene encodes for either methionine (M = ATG) or valine (V = GTG) and is a known genetic factor that contributes to the risk of developing vCJD.[30,38]

In cattle, the western blot profile of C-BSE differs from that of atypical BSE. Atypical BSE cases are designated as such due to the observable variation in molecular mass profiles. More specifically, the molecular mass of the unglycosylated band in BSE prion strains is either higher (H-type)[39,40] or lower (L-type)[41] than that of C-BSE. This is represented in our cartoon representation in Figure 1, reflecting data presented by Wadsworth and Collinge[42] and Biacabe and associates.[39] Moreover, atypical BSE can display a number of additional differences in the disease characteristics, for example, the presence of amyloid plaques associated with L-type BSE also referred to as bovine amyloidotic spongiform encephalopathy (BASE).[41,43] Furthermore, atypical BSE, collectively, exhibit different incubation times[28,44,45] in comparison to C-BSE upon experimental transmission in cattle.

Biological Models for the Study of Prion Diseases

For ethical reasons, animal models are utilized in the study of prion disease transmission, inheritance, route of exposure, progression, and propagation. While nonhuman primates were traditionally thought to be a better model for human disease, the mouse is arguably the most common animal model used by TSE scientists in understanding prion disease, strain differences, and species barriers. Originally, researchers used wild-type mice to examine the many facets of prion disease characterization and propagation.[46–48] However, some of these studies required multiple passages of the specific prion strains to confirm the strain or disease. Although this method is effective at quantifying levels of infectivity of biological samples, it is time consuming and therefore not efficient. Now, transgenic (Tg) mice which overexpress the PrP gene are employed that have distinct advantages as the progression of disease is accelerated as have been observed as a decrease in disease incubation period relative to wild-type mice. It has been noted, again through the use of the mouse model, that prion diseases exhibit a species barrier to transmission.[48–50] Therefore, a variety of Tg mice have been generated, for a number of different species, as a biological tool for each prion disease. For example, Tg mice that express the human prion protein (TgHu) are used in studies characterizing human prion diseases as well as infectivity of cross-species strains. Additional Tg mice include TgOvPrP (sheep), TgBovPrP (cattle), and TgCerPrP (deer), with multiple lines of each species, representing the various commonly identified amino acid substitutions.[51,52] These models can have single or multiple copies of the gene and are extremely useful for strain typing new unknown and uncharacterized natural isolates and for characterizing strain-specific characteristics of prion diseases as well as the various prion strains capacity or potential ability to cross species barriers. The accumulated knowledge and characterization of strains and the acceleration of this accumulated knowledge attributable to the development and use of Tg mice cannot be overstated as this work has advanced our scientific understanding of TSE disease immensely. Additionally, and very importantly in cattle, much advancement in our current understanding of disease acquisition and susceptibility to BSE

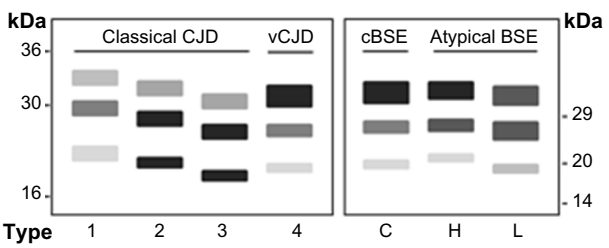

Figure 1. Molecular strain type profiles of human CJD and BSE in cattle.

has been affiliated with the examination of genetic variation. Some of this includes work by the authors of this review and is outlined in the forthcoming section.

Genetic Variations of *PRNP* in Cattle

The prion gene, *PRNP*, in cattle is located on the forward strand of chromosome 13, from 47,400,413 to 47,418,507 base pairs (bp). In cattle, the prion gene consists of three exons, as compared to the human gene which is composed of two exons. However, consistent in all orthologs across species, only the last exon is translated to a protein. There are three genes within this chromosomal locus, the prion gene *PRNP*, the doppel gene *PRND*, and the testis-specific alternatively spliced transcription product *PRNT*. The doppel gene *PRND*, also called prion-like gene (prion protein 2), is located immediately downstream (47,444,352–47,449,390 bp) of the *PRNP* gene. Interestingly, the doppel protein, Dpl, despite its genomic proximity to the *PRNP* gene is not expressed at appreciable levels in the brain; however, it is expressed in the testis and in fact its absence in the testis results in sterility.[16] A third member of this gene locus is prion protein testis–specific *PRNT* found immediately downstream to *PRND* but on the reverse strand. Expression of the *PRNT* gene is exclusively found in the adult testis in human, rhesus monkey, and sheep; however, it is not observed in mouse, rat, and cow.[53]

In addition to the three previously described genes, another gene with a role in prion disease has been discovered. Shadow of prion protein homolog (shadoo) and its gene *SPRN* has been mapped to the reverse strand of chromosome 26, from 25,812,626 to 25,813,057 bp in cattle. Importantly, in addition to the prion gene, *SPRN* has been implicated in prion-related disease susceptibility. Specifically, *SPRN* has been associated with prion disease in cattle[54,55] and humans.[56] *SPRN* is expressed in high levels in the brain and at lower levels in testis.[54] Due to the fact that *SPRN* is more conserved than *PRNP*, it has been hypothesized that it is the ancestor gene of a duplication event. The model proposes that the duplication of *SPRN* gave rise to *SPRNB*, which then resulted in the *PRNP* gene cluster.[53] While there is homology across these genes and some suspected redundancies, the full extent and significance of these relationships have yet to be fully characterized and, of course, merits further study.

Again and somewhat unique to prion diseases, an animal cannot accumulate misfolded proteins, the hallmark of TSE disease, if they do not have that protein to begin with. Therefore, the native prion protein is actually required and essential for the development and progression of TSE disease,[16] and genetic variations in the prion gene have been associated with TSE susceptibility in humans,[1,30] sheep,[57–65] and deer.[66,67] Although amino acid differences in the prion protein are a major contributor to susceptibility and/or resistance risk factor in humans[68] and sheep,[63] this is not the case for cattle. Bovine codon E211K, analogous to codon E200K in human CJD,[68] has only been observed twice in cattle, the first of these cases is associated to atypical BSE and the other case is the offspring of the first.[28,29,69] However, studies in cattle revealed that regions outside of the open reading frame are associated with variation in disease susceptibility. While a few studies identified the octapeptide-repeat region,[70,71] genetic analyses primarily identified two insertion/deletions in promoter regions of the prion gene,[72–77] associated with BSE susceptibility and/or resistance.

Two bovine *PRNP* alleles have been associated with susceptibility to C-BSE: a 23-bp deletion within the promoter region and a 12-bp deletion within intron 1.[72–77] However, the deletion alleles are not entirely independent of one another as there is high linkage disequilibrium (LD) between the two polymorphic sites in *Bos taurus* cattle populations.[78] This suggests that the possible effects of variations in the *PRNP* gene on incidence of C-BSE may be better understood if *PRNP* haplotypes were considered in testing for association with disease incidence. Moreover, *PRNP* haplotypes, containing one or both of the two insertion/deletion alleles, may have a stronger association with either susceptibility or resistance to C-BSE than if the insertions and deletions (indels) are considered independently.

The bovine prion gene contains more than 390 SNPs in the 25-kb region of chromosome 13 containing the *PRNP* gene.[78] This chromosomal segment contains distinct regions of high and low LD that is conserved across many *B. taurus* cattle populations.[78] The region of high LD includes the promoter region, exons 1 and 2, and part of intron 2 (6.7 kb) of the *PRNP* gene. Importantly, both the 23- and 12-bp indels that have been associated with C-BSE susceptibility are contained in this region of high LD.[72–77] The remainder of *PRNP*, including the entire coding region, has relatively low LD. To account for the genetic architecture of the *PRNP* gene, a set of haplotype-tagging single-nucleotide polymorphisms (htSNPs) has been described that efficiently define haplotypes within and across each of the LD regions.[78] These htSNPs were used to test for association between *PRNP* haplotypes and susceptibility to either C-BSE or atypical BSE susceptibility.[76,79] A study conducted by Murdoch and associates examined these htSNPs and the 12-bp and 23-bp indels to test *PRNP* haplotypes for an association with C-BSE in 330 European Holstein cows from the U.K. BSE epidemic, of which 146 were BSE cases and 184 were controls.[76] A combination of sequencing, SNP assay (Illumina goldengate assay), and polymerase chain reaction amplification was used to genotype 18 htSNPs and 2 indels in 95 BSE case and 134 control animals (see Table 1 and Fig. 2, modified from Murdoch et al).[76]

Murdoch et al.[76] noted that some of the SNPs identified in the haplotype (SNP 1392, 1576, and 6811) did not segregate in the test population (Holstein). As a result, the analysis was repeated with a reduced haplotype, and as a result, two SNPs and the insertion indels were identified as significantly overrepresented in animals without disease (Table 2, modified from

Table 1. Analysis of haplotype network in *PRNP*.

HAPLOTYPE	FREQUENCY OF BSE AFFECTED	FREQUENCY OF UN-AFFECTED	CHI SQUARE	*P* VALUE
G C C A I C I A T A	0.132	0.130	0.00161	0.968
T C C G D C D G C A	0.542	0.481	0.843	0.359
G C C A I C I G T A	0.0185	0.0156	0.0264	0.871
T C C G D C D A C A	0.0629	0.0167	2.616	0.106
T C C G D C I G C A	0.0135	0.0223	0.2707	0.603
T C C A D C I G C A	0.0355	0.0472	0.203	0.652
T C C A D C D G C A	0.0561	0.0491	0.0538	0.817
G C C A I T I A C A	0.0332	0.123	7.866	0.005**
G C C A I C I G C A	0.0554	0.0934	1.292	0.256
G C C A I C I A C A	0.0506	0.0214	1.207	0.272

Notes: Haplotype block for SNP 449, 1392, 1576, 1701, InDel 23, 4136, InDel 12, 4731, 4776, 6811. **denotes $P = 0.005$.
Modified from: Murdoch BM, Clawson ML, Yue S, et al. PRNP haplotype associated with classical BSE incidence in European Holstein cattle. PLoS One. 2010;5(9):e12786. Presented here under the terms of the Creative Commons Attribution License.

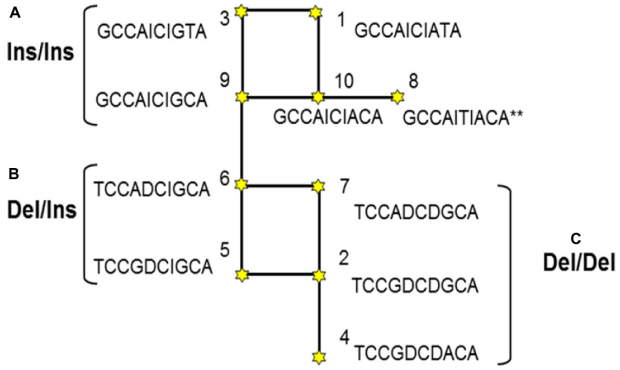

Figure 2. Median joining network haplotypes in *PRNP*. (**A**) Insertion alleles for both 23- and 12-bp indel. (**B**) Deletion allele at 23-bp and insertion allele at 12 bp. (**C**) Deletion allele for both indels.
Note: $**P = 0.005$.
Modified from: Murdoch BM, Clawson ML, Yue S, et al. PRNP haplotype associated with classical BSE incidence in European Holstein cattle. PLoS One. 2010;5(9):e12786. Presented here under the terms of the Creative Commons Attribution License.

Figure 3. Putative transcription factor–binding sites for htSNP 4136.
Note: | denotes where the sequence differs between alleles.
Modified from: Murdoch BM, Clawson ML, Yue S, et al. PRNP haplotype associated with classical BSE incidence in European Holstein cattle. PLoS One. 2010;5(9):e12786. Presented here under the terms of the Creative Commons Attribution License.

Murdoch et al).[76] Upon *in silico* analyses of these comparative haplotype sequences (Fig. 3, modified from Murdoch et al),[76] we hypothesized that sequence variation may ultimately affect protein transcription.

Disease Association Linked to Genes Other than PRNP

To date, studies have shown that the prion gene (*PRNP*), in humans, mice, sheep, and cattle, is the single largest determinant in prion disease susceptibility. However, given the large number of prion protein interactive partners, it is plausible that additional genes contribute to overall susceptibility of disease. A number of *genome-wide association studies* (GWAS) that have been conducted in humans,[80] mice,[81–83] and cattle[84,85] have implicated multiple genes, in addition to *PRNP*, that confer varying degrees of susceptibility, to a variety of prion disease.

It is estimated that a large number of people in the United Kingdom were exposed to BSE-contaminated food during the BSE epidemic; however, to date fortunately only a relatively small number of people have been diagnosed with vCJD. Furthermore, because of the variable and long incubation period of prion diseases, studies intended to follow this population have suggested the actual prevalence of prion infection to be higher. For example, surgically removed tonsils and appendix tissues examined for PrP^{Res} in this population estimate the prevalence to be approximately 1 per 1,000[86,87] to 1 per 2,000.[88] This number is much greater than the number of vCJD cases, and while it fails to recognize the reason, there may be additional factors that can contribute to disease. This suggests that others may still go on to develop the disease.

Table 2. Haplotype analysis with reduced htSNP and indel.

HAPLOTYPE	FREQUENCY OF BSE AFFECTED	FREQUENCY OF UN-AFFECTED	CHI SQUARE	*P* VALUE
A I T I	0.0373	0.173	14.90	0.000114***
A I C I	0.246	0.236	0.0340	0.854
A D C I	0.0417	0.0448	0.0142	0.905
G D C I	0.0173	0.0234	0.118	0.731
A D C D	0.0760	0.0688	0.0446	0.833
G D C D	0.582	0.454	3.938	0.0472*

Notes: Haplotype for SNP 1701, InDel 23, 4136, InDel 12. *denotes $P < 0.05$ and ***denotes $P < 0.001$.
Modified from: Murdoch BM, Clawson ML, Yue S, et al. PRNP haplotype associated with classical BSE incidence in European Holstein cattle. PLoS One. 2010;5(9):e12786. Presented here under the terms of the Creative Commons Attribution License.

Although a small numbers of human cases does not facilitate a powerful study design, efforts have been made to test genomic regions in afflicted humans, in addition to the prion gene region, for disease susceptibility.[80] Despite the limitations, this work has identified genomic regions associated with vCJD disease incidence (RARB and upstream of STMN2) and was later confirmed by some of the same investigators in a larger study.[89] Furthermore, a more recent study did not observe any association with the rare occurrence of *PRNP* gene duplication with sporadic CJD disease incidence.[90] While a number of genomic regions are associated with a variety of prion diseases, the effect of these regions is considered modest and to date the prion gene itself is the strongest risk factor across human prion diseases, though interestingly, some of genomic regions associated with the occurrence of vCJD overlap with syntenic regions identified in animal studies. Although this may be purely coincidental, it is certainly noteworthy given the cross-species applicability of BSE and vCJD.

GWASs in cattle have been conducted from cattle involved in the U.K. BSE epidemic. Given that it was an actual outbreak rather than a controlled experiment, it is not possible to determine and thus control for the levels of exposure to infectious material. For example, exposure to a large enough dose of infectious material will result in disease. Further, these study designs are often limited in power for a number of different reasons. First the number of samples required for population-based analyses is all too often limiting. Second, the information and design of SNP assays has limitations. For example, the number, the minor allele frequency, and the extend of linkage disequilibrium of the SNPs can differ in different populations. These factors all contribute to a decrease in the sensitivity and power and accordingly the ability to detect association. Regardless of these aforementioned challenges, a number of genomic regions that influence susceptibility to BSE have been observed in cattle.

To test for associations with BSE susceptibility, a genome-wide scan using a panel of 3,072 SNP markers on 814 animals representing cases and control Holstein cattle collected in the mid-1990s in Southern England during the U.K. BSE epidemic was employed by the authors and our collaborators.[84] The Holstein case and control cattle analyzed consisted of two sets, one set with known family relationships and the second set of multiple families with BSE cases with paired controls. The family set comprising half-sibling progeny from six sires revealed 27 SNPs representing 18 chromosomes associated with the incidence of BSE disease, confirming previously reported chromosomal regions. A higher resolution, 50,000-SNP, genome-wide scan was performed using the second set, including 143 BSE-affected (case) and 173 unaffected half-sibling (control) animals, to test for association with BSE incidence.[85] The data analysis identified loci on two different chromosomes that are associated with BSE disease occurrence. The most significant association is an SNP on chromosome 1 at 29.15 Mb that is associated with BSE disease. Additionally, a locus on chromosome 14, within a cluster of SNPs trended toward significance. It is worth noting that there is a region of chromosome 8 identified in human vCJD study[80] that shares synteny to the region identified on cattle chromosome 14 associated with the disease. Further, candidate genes appear to have plausible biological relevance with the known etiology of TSE disease. Comparative mapping identified one of the candidate gene, hypothetical gene LOC521010, similar to FK506-binding protein 2 located on chromosome 1 at 29.32 Mb.[85] This gene encodes a protein that is a member of the immunophilin protein family and is involved in basic cellular processes including protein folding. The chromosomal regions identified and the candidate genes within these regions merit further investigation.

Summary

So what do we know about BSE? We know that BSE, like all TSE, is a disease of protein misfolding. It is this protein misfolding of a native endogenous protein that switches the primary conformation, in the case of BSE, from α-helices into a β-pleated sheet secondary protein structure. Furthermore, we know that this misfolded prion protein is proteinase K resistant, as such can accumulate, and is in some part responsible for the symptomology and progression of prion disease. We know that the native prion protein is coded for by the *PRNP* gene and that genetic variation in this gene, particularly those linked to an amino acid substitution, can affect susceptibility to disease. We know that transmission and development of prion disease

can occur in several ways and, not surprisingly, the largest determinant to acquiring a prion disease is exposure to infectious material. Simply put, exposure of susceptible animals to enough infectious material will cause disease, as demonstrated in a variety of animal models. We know that different prion strains exist and influence the disease profile. Importantly, we have determined that improved management of SRM, namely bone meal, blood meal, and neural tissue, reduces incidence of BSE. We think that BSE is unlikely to be transmitted through either horizontal or vertical routes of transmission and there is good evidence to support this. We know genes other than *PRNP* in cattle are associated with risk of acquiring prion disease including the prion family member *SPRN*. Furthermore, it was discovered that other genomic regions including regions of indel can impact the risk of developing prion disease.

We know these things because of decades of research using multiple techniques including histology, immunohistochemistry, western blotting, genomic sequencing, GWAS, *in silico* sequence analyses, Tg animal studies, and long-term cross-species infectivity studies. Most importantly, what we know for certain is that we do not know everything. Therefore, much work remains in order for the scientific community to fully understand how cross-species prion diseases are acquired and to truly understand the full nature of how prion diseases develop and progress. There are reports of a sheep presumed to be resistant to scrapie[91] exhibiting disease as well as identification of new strains,[92] which poses the very important question, how do new prion strains develop and can prion strains change? Importantly, a recent report by Cassard and colleagues[93] has provided evidence implicating different scrapie strains with human zoonotic properties, to the most abundant human CJD, sporadic CJD. This study was able to demonstrate scrapie infection of tgHu mice upon second passage developed into disease with the same molecular profile as that of sporadic CJD. While this study warrants further investigation, the implications of another route to jumping the species barrier is profound. The authors hope that this review makes readers aware of some of the progress made towards understanding TSEs, especially BSE. Moreover, there remains substantial value in performing additional research, identifying improved methods of antemortem testing. We would benefit from having a better understanding of actual resistance to prion disease acquisition, as recently demonstrated PrP V127 identified to confer complete resistance in humans,[94] versus longer incubation times, improved characterization of the sources of infectivity, the manner by which some prion diseases cross species barriers, how they occur spontaneously, and why they progress at different rates. Finally, it would be beneficial to acquire sufficient understanding of all TSEs to allow for development of effective treatments. For all these reasons and the inherent love of science and discovery, the authors strongly encourage members of the research community to remain persistent in their pursuit of prion research, remain committed to an improved understanding of all TSEs, and remain diligent in their distribution of new discoveries and refinement of current hypotheses surrounding all aspects related to prion-mediated diseases.

Author Contributions

Wrote the first draft of the manuscript: BMM. Contributed to the writing of the manuscript: BMM, GKM. Agree with manuscript results and conclusions: BMM, GKM. Jointly developed the structure and arguments for the paper: BMM, GKM. Made critical revisions and approved final version: BMM, GKM. Both authors reviewed and approved of the final manuscript.

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