Understanding Intra-Species and Inter-Species Prion Conversion and Zoonotic Potential Using Protein Misfolding Cyclic Amplification

Citation for published version:
Peden, AH, Suleiman, S & Barria, MA 2021, 'Understanding Intra-Species and Inter-Species Prion Conversion and Zoonotic Potential Using Protein Misfolding Cyclic Amplification', Frontiers in Aging Neuroscience, vol. 13. https://doi.org/10.3389/fnagi.2021.716452

Digital Object Identifier (DOI):
10.3389/fnagi.2021.716452

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Frontiers in Aging Neuroscience

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Understanding Intra-Species and Inter-Species Prion Conversion and Zoonotic Potential Using Protein Misfolding Cyclic Amplification

Alexander H. Peden, Suzanne Suleiman and Marcelo A. Barria*

National CJD Research & Surveillance Unit, Centre for Clinical Brain Sciences, Deanery of Clinical Medicine, The University of Edinburgh, Edinburgh, United Kingdom

Prion diseases are fatal neurodegenerative disorders that affect humans and animals, and can also be transmitted from animals to humans. A fundamental event in prion disease pathogenesis is the conversion of normal host prion protein (PrP\textsuperscript{C}) to a disease-associated misfolded form (PrP\textsuperscript{Sc}). Whether or not an animal prion disease can infect humans cannot be determined a priori. There is a consensus that classical bovine spongiform encephalopathy (C-type BSE) in cattle transmits to humans, and that classical sheep scrapie is of little or no risk to human health. However, the zoonotic potential of more recently identified animal prion diseases, such as atypical scrapie, H-type and L-type BSE and chronic wasting disease (CWD) in cervids, remains an open question. Important components of the zoonotic barrier are (i) physiological differences between humans and the animal in question, (ii) amino acid sequence differences of the animal and human PrP\textsuperscript{C}, and (iii) the animal prion strain, enciphered in the conformation of PrP\textsuperscript{Sc}. Historically, the direct inoculation of experimental animals has provided essential information on the transmissibility and compatibility of prion strains. More recently, cell-free molecular conversion assays have been used to examine the molecular compatibility on prion replication and zoonotic potential. One such assay is Protein Misfolding Cyclic Amplification (PMCA), in which a small amount of infected tissue homogenate, containing PrP\textsuperscript{Sc}, is added as a seed to an excess of normal tissue homogenate containing PrP\textsuperscript{C}, and prion conversion is accelerated by cycles of incubation and ultrasonication. PMCA has been used to measure the molecular feasibility of prion transmission in a range of scenarios using genotypically homologous and heterologous combinations of PrP\textsuperscript{Sc} seed and PrP\textsuperscript{C} substrate. Furthermore, this method can be used to speculate on the molecular profile of PrP\textsuperscript{Sc} that might arise from a zoonotic transmission. We discuss the experimental approaches that have been used to model both the intra- and inter-species molecular compatibility of prions, and the factors affecting PrP\textsuperscript{C} to PrP\textsuperscript{Sc} conversion and zoonotic potential. We conclude that cell-free prion protein conversion assays, especially PMCA, are useful, rapid and low-cost approaches for elucidating the mechanisms of prion propagation and assessing the risk of animal prions to humans.

Keywords: neurodegenerative disease, prion, CJD (Creutzfeldt-Jakob disease), protein misfolding, in vitro aggregation assays, surveillance, zoonosis, prion diseases
OVERVIEW

Prion

Prions – *Proteaceous infectious particles* – are self-propagating structures identified as the major or sole cause of prion diseases, which are lethal neurodegenerative disorders that affect animals and humans. The pathogenesis of prion diseases is strongly believed to be related to protein misfolding. The normal prion protein, PrPC, encoded by PRNP, is a highly conserved cell surface glycoprotein. It is thought to have a number of physiological roles in both the central and peripheral nervous system and may serve as a regulatory protein in cell proliferation and differentiation (Halliez et al., 2014; Wulf et al., 2017). In prion disorders, PrPC is believed to change its conformation and become misfolded. The abnormal misfolded version, PrPSc, is partly resistant to proteinase K and can self-reproduce by causing PrPC to convert to its abnormal isoform. The resultant misfolded molecules form multi-chain fibrillary/amyloid aggregates. Over months to several years in vivo, these structures accumulate in brain and nerve tissue, triggering cell death and the neurodegeneration associated with prion diseases (Prusiner, 1982; Ironside et al., 2014; Love et al., 2015).

Animal and Human Prion Diseases

Animal prion diseases include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, transmissible mink encephalopathy (TME) and chronic wasting disease (CWD) in several cervid species, including, elk, moose, and reindeer or caribou (Sigurdson and Aguzzi, 2007). More recently, a novel type of prion disease was reported in dromedary camels (Babelhadj et al., 2018). In humans, prion diseases are clinically and pathologically heterogeneous, and can present as either (1) spontaneous disorders, e.g., sporadic CJD (sCJD) and variably protestase-sensitive prionopathy (VPSPr); (2) genetic disorders, including Gerstmann-Sträussler-Scheinker disease (GSS) and genetic CJD (gCJD) (Richt and Hall, 2008); (3) and acquired/transmissible forms, such as variant CJD (vCJD), iatrogenic CJD (iCJD) and kuru. The transmissible neurodegenerative diseases caused by prions are also known collectively as “transmissible spongiform encephalopathies” or TSEs.

Prion Strains

Currently, the protein-only hypothesis remains the most accepted paradigm in prion research. In this model, prions are transmissible particles that are devoid of nucleic acid and seem to be composed entirely of a modified disease-associated form of the prion protein (PrPSc) (Griffith, 1967; Prusiner, 1982). In this model PrPSc self-propagates by conversion of PrPC and is able to infect new individuals and sometimes, as in BSE, is able to transmit to a different species. However, PrPSc still resembles other conventional infectious agents by expressing characteristic strain variations; i.e., a particular prion strain can encode one of various specific disease phenotypes that are maintained after inoculation and serial passage in experimental animals. It was initially assumed that the strain phenomenon might be a function of the PrPSc primary structure of the host. However, different prion strains were also identified in genetically identical mice suggesting that strain variation is not simply dependent on amino acid similarities between donor and recipient (Collinge and Clarke, 2007). According to the protein-only hypothesis the strain-specific properties are enciphered in the various conformations that PrPSc can adopt and are maintained when PrPSc replicates through conversion of PrPC (Griffith, 1967; Prusiner, 1982).

Prion diversity has historically been investigated by experimental transmission studies in animal models, where several criteria, including the length of time between inoculation and appearance of clinical signs, the type of clinical signs, neuropathological changes (score profile) and biophysical measures can be measured (Brandner and Jaunmuktane, 2017; Igel-Egalon et al., 2018). The classical way of biochemically classifying prion diversity is by PrPSc proteolysis and studying the biochemical profile of the protease resistant core on western blot analysis. Three major bands are usually observed upon cleavage with proteinase K, corresponding to un-, mono- and di-glycosylated forms of PrP. The size and relative abundance of these three forms is usually characteristic of a particular prion variant, referred to as the PrPres subtype (Head and Ironside, 2012). In the classification of human prion disease subtypes using the nomenclature of Parchi (Parchi et al., 2012), unglycosylated bands at 21 or 19 kDa are associated with PrPSc types 1 and 2, respectively. In addition, a predominance of either the mono- or di-glycosylated band is assigned as type A and B, respectively. All sporadic CJD cases are of the ‘A’ glycoform and are either type 1 or 2, whereas variant CJD is associated with type 2B. These differences in fragment size and glycosylation ratios are believed to be associated with different PrPSc conformations, which are in turn associated with different prion strains.

Polymorphic differences clearly play a key role in influencing prion strains in human prion disease. It is well-recognized that codon 129 of the prion protein gene PRNP, which has either methionine (M) or valine (V), correlates with the molecular typing and prion strain characteristics (Love et al., 2015). The strain phenomenon is observed in sporadic forms of prion disease. Sporadic CJD Type 1 subtype is favored in individuals who are methionine homozygous (MM) at polymorphic codon 129 of PRNP, whereas valine homozygosity at this codon favors Type 2. MV heterozygous individuals with sCJD are more evenly split between Type 1 and Type 2 (Parchi et al., 1999). The association of sCJD subtypes with strains was supported by transmission studies to transgenic mice expressing human PRNP (TgHu) with the three PRNP codon 129 genotypes (MM, MV, and VV), which suggested the existence of four prion strains (M1, M2, V1, and V2), and a fifth strain corresponding to a thalamic sub-subtype, MM2T, also known as sporadic fatal insomnia (Bishop et al., 2010; Moda et al., 2012). However, the example of sCJD subtypes alludes to the existence of an overlap between prion strains, with the commonly observed co-existence of more than one PrPSc subtype within individual sCJD patients (Polymenidou et al., 2005; Uro-Coste et al., 2008; Cäli et al., 2009; Parchi et al., 2009).
Conformation Selection Theory

In light of evidence that prion strains may not be absolutely discreet entities, Collinge and Clarke (2007) proposed a conformation selection theory in which a prion strain is thought to be comprised of not just one type of PrP\textsuperscript{Sc} molecular assembly, but instead a range or cloud of assemblies with different tertiary and quaternary structures. In this model, a subset of assemblies within this cloud may be selected for replication in a new individual of the same species with an alternate PRNP genotype, or in a new species. The transmission will occur more readily if a significant subset of conformers of the prion strain can successfully replicate in the new host, and this will be determined in part by the compatibility of the PrP sequence.

Development of in vitro Models as Versatile and Reproducible Alternatives to Investigate Prion Diseases

Evaluation of transmission barriers using the in vivo models requires the use of large numbers of experimental animals. In addition, despite the development of the transgenic animal models, the speed with which animal research can be conducted has been hindered by long incubation periods, especially when serial passage is being used to evaluate prion strain stabilization or adaptation. Therefore, there has been an urgent need to develop alternative, quicker, more cost-efficient and reliable in vitro models.

The first in vitro cell-free system for modeling prion conversion was demonstrated by Kocisko et al. (1994). They showed that the radiolabeled recombinant hamster PrP\textsuperscript{Sc} was able to selectively convert to its PK-resistant PrP\textsuperscript{Sc} isoform in the presence of unlabeled hamster PrP\textsuperscript{Sc}. This conversion occurred even after de-glycosylation of PrP\textsuperscript{Sc} or transient denaturation with a chaotropic agent (GdnHCl) (Kocisko et al., 1994).

Protein Misfolding Cyclic Amplification (PMCA)

A few years later, Saborio et al., 2001 successfully established a procedure that applied cycles of incubation and sonication to efficiently accelerate the conversion of PrP\textsuperscript{C} in a hamster brain homogenate by hamster PrP\textsuperscript{Sc}. The process was named Protein Misfolding Cyclic Amplification, or PMCA (Saborio et al., 2001; Figure 1). In the seeding phase of PMCA, an excess amount of PrP\textsuperscript{Sc} (termed the substrate) is seeded with small quantities of PrP\textsuperscript{Sc} (termed the seed). During the amplification phase, the mixture is incubated at 37°C and the PrP\textsuperscript{Sc} seed induces the generation of large PrP\textsuperscript{Sc} aggregates at the expense of PrP\textsuperscript{C} (Figure 1). PMCA thus mimics the natural propagation of PrP\textsuperscript{Sc} and its aggregation in the brains of TSE-infected individuals. In order to increase amplification efficiency, ultrasound power is applied periodically to break down these large misfolded aggregates and produce more of the active converting units, which accelerates the reaction in an exponential manner. By the end of one round of PMCA, most molecules are present in their misfolded conformation and PrP\textsuperscript{Sc} becomes detectable. In the detection phase of PMCA, the amplified product is detected, usually by western blotting following limited proteolysis to detect the protease resistant fragments of PrP\textsuperscript{Sc} (PrPres) (Figure 2). Several studies that followed the original demonstration of PMCA indicated the flexibility, fidelity and reliability of this method. PMCA was shown to reproduce the infected agent in vitro by serially repeating the amplification round on the diluted product (Castilla et al., 2008b, 2005). The infectivity per unit of PMCA PrP\textsuperscript{Sc} was comparable to that of PrP\textsuperscript{Sc} from the infected tissue used as the seed (Castilla et al., 2005). The serially amplified product, which becomes devoid of the original seed, was shown to hold a comparable secondary structure, electrophoretic mobility and glycosylation profile to that of the seed (Castilla et al., 2005). Moreover, strain-specific infectivity was revealed to be conserved within the amplified product when...
Peden et al. Understanding Intra-Species and Inter-Species Prion Conversion

FIGURE 2 | Protein misfolding cyclic amplification and its three technical phases. In designing a PMCA experiment there are three phases to consider, which each involve decisions depending on the research question and resources available. In the seeding phase, a minute amount of PrP\textsuperscript{Sc} seed (small green circle) is mixed with an excess of PrP\textsuperscript{C} substrate (large brown circle). For both these components, the species, PRNP genotype, and tissue source need to be chosen. In addition, the prion strain of the seed needs to be selected. In the amplification phase, cofactors may be added to the above mixture which is then subjected to cycles of incubation and sonication. Choices are required for the physical parameters (e.g., cycle time, incubation versus sonication time per cycle, number of cycles, and amplitude of ultra-sonication). In the detection phase, the amplified product is quantified and/or characterized, usually by western blotting following limited protease digestion. Cross-species amplification can be confirmed with species-specific detection antibodies, whereas alterations in the PrP\textsuperscript{res} molecular profile can be confirmed with subtype-specific antibodies.

four mouse-adapted scrapie (RML, ME7, 139A, 79A), one mouse adapted BSE (310C) and four human prion disease (vCJD, sCJD MM1, sCJD MM2, sCJD VV2) PMCA-propagated strains were inoculated into experimental animals. Indeed, after serial rounds of PMCA, the PMCA-amplified product caused a comparable disease phenotype in experimental animals (incubation time, clinical signs and neuropathological profile) as the original seed (Castilla et al., 2008b; Cali et al., 2019). Thus, the characteristics of the original strain used as seed seems to be still encoded within the PMCA amplified product, providing fundamental proof that the strain characteristics, including infectivity, are preserved after in vitro amplification. In only a few weeks this technique can replicate PrP\textsuperscript{Sc} and conserve prion strain properties in a manner that recapitulates the time-consuming in vivo approach that might take several years.

The development of in vitro conversion assays, such as PMCA, opened a new era in prion research. In time, improvements in the sensitive of PMCA allowed the detection of extremely low levels of PrP\textsuperscript{Sc} in peripheral tissues and biological fluids in human samples (Moda et al., 2014; Bougard et al., 2016, 2018; Concha-Marambio et al., 2016; Barria et al., 2018a; Concha-Marambio et al., 2020).

UTILIZATION OF PMCA TO INVESTIGATE HOMOLOGOUS MOLECULAR COMPATIBILITY ASSOCIATED WITH PRIONS CONVERSION

As mentioned before, in humans, prion strain variation and the polymorphic codon 129 of PRNP are two of the major biological determinants of the clinicopathological phenotype of the disease and an individual's susceptibility to develop prion diseases. PRNP polymorphism imposes its effect by altering prion strain conformation. Empirical evidence for this came from Parchi et al. (2000) who showed that codon 129 is a primary determinant of the proteinase cleavage site of PrP\textsuperscript{Sc} in CJD, resulting in a change in the size of the PrP proteinase resistant fragments (Parchi et al., 2000).

Although allelic variation percentages for this codon vary across different ethnic groups, homozygosity of methionine is a major risk factor in all CJD types (Deslys et al., 1998). Indeed, with the exception of one case, all clinical and pathologically confirmed vCJD cases, including three cases of secondary vCJD following blood transfusion, have had an MM genotype at codon
129. The exception is the last reported United Kingdom case that was MV at codon 129 (Mok et al., 2017). Similarly, susceptibility to sCJD is considerably higher in methionine homozygous individuals (Palmer et al., 1991). In addition, sporadic CJD patients who are codon 129 heterozygous have longer durations of disease (Pocchiari et al., 2004).

To shed more light on the role of codon 129 polymorphism on prion strain characteristics and transmissibility, Jones et al., 2007 recapitulated the transmission properties of vCJD and sCJD in two important PMCA studies. The first employed PMCA to model secondary vCJD transmission in humans (Jones et al., 2007). This study used either healthy human brain tissues or humanized transgenic mice models expressing the three possible codon 129 genotypes (MM, MV, and VV). Interestingly, vCJD propagated efficiently in both MM substrates, supporting the idea that codon 129 has a strong influence on PrP\textsuperscript{Sc} conversion and infectivity. Less amplification was observed in MV2 transgenic mouse brain tissues, while no amplification was observed in VV substrates, which suggested that this genotype might be a protective factor.

This study emphasized the impact of PRNP codon 129 genotype on susceptibility to human-to-human transmission of vCJD and indicated that MM imposes a higher risk than the other two genotypes. MV individuals still have some risk of developing vCJD and might remain asymptomatic for long periods before developing clinical disease.

The second PMCA study by Jones et al. used (MM/MV/VV) homogenates as substrates and six sCJD disease PrP\textsuperscript{Sc} seeds representing the six phenotypic subtypes based on subtype and codon 129 genotype: MM1/MV1, VV1, MM2 cortical, MM2 thalamic, MV2 and VV2 (Jones et al., 2008). As with vCJD, the results indicated that PMCA was an attractive model for investigating the molecular basis of phenotypic variability in sCJD. A considerable degree of compatibility in terms of the PrP\textsuperscript{Sc} subtype was observed between the sCJD seed and the PMCA product. Here too, codon 129 polymorphism was shown to have an impact on amplification efficiency, which reflected the relationship observed between PrP\textsuperscript{Sc} subtype and codon 129 genotype in sCJD patients. Successful amplification was recorded when seed and substrate codon 129 polymorphisms were matched. Remarkably, MV1 resembled MM1 as it tended to amplify better with MM substrate, whereas MV2 preferred VV substrate. Therefore, the substrate specificity of PrP\textsuperscript{Sc} from MV sCJD patients appeared to depend on PrP\textsuperscript{Sc} type.

**Iatrogenic Creutzfeldt-Jakob Disease: Molecular Approaches**

Acquired forms of CJD caused by prion infection as a result of medical or surgical interventions are collectively referred to as iatrogenic CJD (iCJD). Apart from secondary transmission of vCJD via blood or blood products (Peden et al., 2004; Urwin et al., 2016), iCJD also includes cases acquired by the use of contaminated medical instruments (Bernoulli et al., 1977; Will and Matthews, 1982), transplantation of contaminated corneal grafts (Duffy et al., 1974), cadaveric dura mater (DM-iCJD) (Brown et al., 2012), or treatment with contaminated cadaveric growth hormone (GH-iCJD) (Gibbs et al., 1985; Koch et al., 1985; Powell-Jackson et al., 1985; Ritchie et al., 2017). DM-iCJD and GH-iCJD comprise the majority of iCJD cases worldwide (Brown et al., 2012; Ritchie and Barria, 2021).

The PRNP codon 129 genotype affects susceptibility and disease phenotype in iCJD. Surveillance studies on a French cohort indicated that the majority of the GH-iCJD cases were codon 129 methionine homozygous (Delisle et al., 1993; Brandel et al., 2003). In contrast, where codon 129 data is available most United Kingdom cases of iCJD are either heterozygous (MV), or valine homozygous (VV) and only a low proportion ~10% were homozygous for methionine (MM) (Brandel et al., 2003; Rudge et al., 2015; Ritchie et al., 2017). This suggests that original sources of contamination for the United Kingdom and French GH-iCJD epidemics were of different prion strains. The MM cases tended to occur later in United Kingdom GH-iCJD epidemic, suggesting a longer incubation period for patients with this genotype, which may indicate a genotypic barrier in these individuals to the contaminating prion strain. Alternatively, the MM cases may be the result of infection from a minor component of a mixture of contaminating strains.

Although the use of cell-free assays to model iatrogenic CJD is limited, PMCA was used in combination with other biochemical analyses to investigate the prion strain origin of the United Kingdom GH-iCJD epidemic, and the possibility that MM GH-iCJD cases might exhibit the traceback phenomenon (Ritchie et al., 2017).

**Traceback Phenomenon**

The traceback phenomenon was observed in animal research studies when sCJD brain inocula of V2 strain was transmitted to humanized mice MM at PRNP codon 129 (Kobayashi et al., 2007). This transmission resulted in a new strain characterized by a unique disease phenotype featuring long incubation periods, and kuru-like plaques on histological examination. In addition, this strain was associated with an altered PrP\textsuperscript{Sc} conformation that caused the appearance of an upshifted proteinase-resistant unglycosylated band at ~20 kDa on western blots, referred to as intermediate PrP\textsuperscript{Sc} (PrP\textsuperscript{Sc} subtype i). When this strain was inoculated on second passage into TgHu VV mice, the PrP\textsuperscript{Sc} appeared to regain its original conformation, resulting in type 2 PrP\textsuperscript{Sc} (Kobayashi et al., 2007). This property suggests that the new strain (PrP\textsuperscript{Sc} i) still retains within its conformational cloud some traits from the original V2 strain that are “inherited,” which means that when transmitted into new animals it can express its original strain characteristics once again (Kobayashi et al., 2009, 2010).

**PMCA Investigation of Traceback Phenomenon**

Two PMCA methods have been used to investigate the traceback phenomenon. Takeuchi et al. (2016) utilized cell-PMCA (where the PrP\textsuperscript{Sc} source was from a 293F cell-lysates, overexpressing the human form of the prion protein) to
amplify brain homogenate samples from two DM-iCJD cases, both MM at codon 129 in Japan. The cases considered for the study included a case of non-plaque-type DM-iCJD (np-dCJD) resembling sCJD MM1/MV1, while the other case was plaque-type DM-iCJD (p-dCJD) characterized by kuru plaques and PrPSc with an intermediate type (PrPSc subtype i). The p-dCJD PrPSc material presented molecular compatibility when a substrate homozygous for valine, not methionine, was used. The amplified material resembled the sCJD V2 strain biochemical typing. These results appear to defy the expected relationship between PMCA efficiency and the genotype compatibility of seed and substrate. The authors suggested this was evidence that plaque-type DM-iCJD was the result of transmission of the V2 CJD strain to codon 129 MM patients, and its efficient amplification in VV substrate was an indication of the traceback phenomenon.

Ritchie et al. (2017) utilized several approaches to model 21 United Kingdom cases of GH-iCJD and three cases of DM-iCJD. In particular, the authors were interested in two GH-iCJD cases homozygous for methionine at codon 129: One featured the plaque subtype and had PrPSc type i (subtype MMi), whereas the other was of type 1 and had no plaques (subtype MM1). Samples of cerebral cortex tissue from these cases were used to seed humanized transgenic mouse brain substrate carrying the codon 129 MM and VV. These two MM GH-iCJD cases gave divergent results. For the MM1 case amplification was observed with the valine, not the methionine, homozygous substrate, whereas the MM1 cases failed to amplify efficiently with either substrate. More importantly, the molecular strain typing profile of the amplified product of the VV substrate seeded with MMi showed a downshifted migration of the proteinase-resistant unglycosylated form by western blotting when compared to the original material. In contrast when MM substrate was used to amplify PrPSc from the MM1 case, an upshift occurred. The amplification of MMi PrPSc with VV substrate and the downshift of the molecular profile is possibly indicative of the traceback phenomenon mentioned earlier; i.e., it may provide indirect evidence that the MMi subtype results from the replication V2 strain in a genotypically mismatched methionine homozygous individual and that this subtype readily reverts to a downshifted, type 2-like molecular profile when provided with a suitable VV substrate for replication.

Both studies strongly indicated that PMCA is a rapid and reliable method for modeling the molecular mechanisms of prion transmission across genotypic barriers and the traceback phenomenon. It would appear to be possible to identify DM-iCJD cases in MM individuals that actually originated from contamination with the V2 sCJD strain, rather than the M1 strain. Additionally, Kobayashi et al. (2016) have suggested that kuru plaque pathology and PrPSc type i in codon 129 MM patients diagnosed with sCJD may actually indicate an acquired or iatrogenic etiology in these patients. Therefore, PMCA could help ascertain the acquired origin of atypical sCJD cases, without having actual evidence of iatrogenic exposure by examining the potential for PrPSc amplification using matched and mismatched humanized substrates (PRNP codon 129 MM or VV).

### UTILIZATION OF PMCA TO INVESTIGATE HETEROLOGOUS MOLECULAR COMPATIBILITY ASSOCIATED WITH PRIONS CONVERSION

#### The Species Barrier

The species barrier refers to the combined factors that limit the ability of a pathogen from one species to cross-infect another species. Zoonotic prion refers to the likelihood this barrier might be breached to infect humans. When examining the barrier that may exist between two species for the transmission of a given prion strain, the species from which the prion strain originates can be considered to be the species of origin. The species whose threat is being assessed (e.g., humans) is the target species. Any other species through which the prion strain might pass between the species of origin and the target species is an intermediate species.

#### Zoonotic Prion Disease Threats to Humans

Bovine spongiform encephalopathy is a prion disease of cattle, first recognized in 1986 in the United Kingdom, reaching epidemic proportions and then being brought under control (Hope, 2013). Dietary exposure to BSE is thought to be the cause of vCJD in humans (Will et al., 1996; Bruce et al., 1997; Hill et al., 1997). The classical form of BSE (also known as C-type BSE, to distinguish it from atypical forms) is therefore currently the only known zoonotic prion disease described.

Scrapie is a prion disease of sheep, endemic in many countries including the United Kingdom, which comprises several distinct strains (Greenlee, 2019). There is polymorphic variation in the sheep PRNP at amino acid positions 136, 154, and 171 that affects susceptibility to scrapie, and the VRQ, ARQ, and ARR genotypes have the greatest effect in this regard (Goldmann, 2008; Gonzalez et al., 2012). Depending on the infecting source or strain, VRQ/VRQ and ARQ/ARQ sheep are the most susceptible to classical scrapie, whereas ARR/ARR is effectively resistant (Hunter and Bossers, 2006). Although scrapie has been known to exist for more than 200 years, in stark contrast to BSE, there is no epidemiological evidence to suggest is poses a threat to human health (Brown, 1998; van Duijn et al., 1998).

A longstanding question is whether BSE can pose a zoonotic threat to humans via sheep. When considering zoonotic potential from animal prion diseases to humans as the target species, the risk posed by BSE from cattle as the species of origin is well known. It remains unknown whether the United Kingdom sheep flock was exposed to BSE early in the BSE epidemic, and whether BSE could propagate and adapt in sheep to resemble scrapie but retain its zoonotic potential for infecting humans. However, various experimental approaches have been taken to model this scenario as described below.

#### BSE in Sheep

Although no natural cases of BSE in sheep have been detected, sheep can be experimentally infected with BSE...
(Foster et al., 2001), and it had been suggested that the clinical signs of scrapie and sheep-BSE are clinically indistinguishable (Stack et al., 2006) and that these forms of prion disease could only be differentiated by careful immunohistochemical examination and biochemical analysis of the protease resistant PrPSc in the central nervous system of affected animals (Jeffrey et al., 2001; Gonzalez et al., 2003; Thuring et al., 2004). It is therefore possible that adaptation of the BSE agent to an ovine host involves a change in the molecular properties of the BSE prions and that this change may have occurred prior to the introduction of statutory testing of sheep for scrapie. An important concern is whether sheep-adapted BSE would retain the potential to infect humans, or indeed be more virulent (Espinosa et al., 2007; Plinston et al., 2014).

**Using Serial PMCA to Assess the Zoonotic Risks**

Many transmission studies have investigated the potential of BSE to infect sheep (Foster et al., 2001; Gonzalez et al., 2003; Stack et al., 2009), and for sustained transmission of BSE in flocks (Bellworthy et al., 2005; Jeffrey et al., 2015), and the potential for BSE-infected sheep to infect humans (Plinston et al., 2014; Joiner et al., 2018). As mentioned previously, a disadvantage of these in vivo investigations is that they are often lengthy and expensive. The *in vitro* conversion assay, PMCA, provides an alternative means for investigating species barriers and for specifically examining the molecular feasibility of zoonotic transmissions. In the serial automated PMCA (saPMCA), the product from one round of PMCA is used to seed fresh PrPSc substrate in successive rounds of PMCA performed in series (Castilla et al., 2005, 2006) (Figure 3). By providing fresh PrPSc substrate with each round, saPMCA overcomes a major limitation of PMCA which is the thermostability of PrPSc at 37°C and thereby extends the amplification process. Therefore, saPMCA can be used to investigate whether the propagation of a prion strain with PrPSc substrate from another species is possible and moreover whether in can be sustained (Castilla et al., 2005; Krejciova et al., 2014). Serial automated PMCA can also be used to investigate whether the propagation of a prion strain from the species of origin via an intermediate species modifies its potential for infecting and propagating in the target species in question, for example humans.

**Investigating Whether a Prion Strain Can Be Continuously Propagated in vitro**

The potential for BSE prions to be continuously propagated in sheep was investigated by saPMCA. Using sheep ARQ/ARQ PrPSc as a substrate, Krejciova et al. (2014) showed that BSE from cattle, or experimentally challenged sheep, could be continuously amplified over at least eight rounds. The PrPSc subtype was unaltered, suggesting a conservation of the BSE agent strain using this substrate. These findings agreed with an animal transmission study showing BSE prions could be serially passaged three times through sheep of the ARQ/ARQ genotype, with only minor alternations of the molecular properties of PrPSc (Stack et al., 2009). In contrast, PrPSc from ARR/ARR sheep, a genotype known to be more resistant to scrapie, could not sustain the amplification of cattle or sheep BSE PrPSc over successive rounds (Krejciova et al., 2014), even though amplification of sheep BSE PrPSc was observed for seeds in the first round.

**Modeling Strain Adaption**

In addition to testing for sustainable prion propagation, saPMCA can also be used to investigate whether the properties of the strain are modified and to explore the possibility of strain adaption in either an intermediate species or the target species. Alterations in the molecular profile of the PrPSc amplified product can be used as indicator of changes to agent strain (Castilla et al., 2008a). In most cases these will be changes in the molecular mass of the unglycosylated fragment or the relative intensities of the di-, mono- and unglycosylated fragments, which can be monitored by analyzing samples of the saPMCA rounds by western blotting.

Changes in the molecular subtype that arise during saPMCA can also be detected using PrPSc subtype-specific antibodies. For instance, when sheep BSE was propagated using ovine VRQ/VRQ substrate by saPMCA, the generic anti-PrP 6H4 monoclonal antibody detected an upward shift to more scrapie-like molecular profile in the latter rounds. This was further verified using the 12B2 monoclonal antibody, which specifically detects the longer, slower migrating protease resistant PrPSc fragment associated with scrapie (Krejciova et al., 2014). This indicates a possible strain adaptation that might occur in vivo if BSE infection spread into a sheep population.

This was not the first study to show that saPMCA could model the process of strain adaptation. For instance, Green et al. (2008) demonstrated that saPMCA could enable mouse prions of the RML strain to adapt to replication using deer PrPSc. When RML prions were propagated using cervid PrPSc expressed in TgCer mice the physiochemical properties and western blot profile of PrPSc were altered, and there was an increased ability to infect TgCer mice resulting in a prion strain with unique properties (Green et al., 2008).

**Mechanisms of Strain Adaption**

The mechanism by which prion strain adaptation occurs *in vitro* or *in vivo* is still at matter of debate. The shift in the molecular profile observed after multiple rounds of saPMCA using PrPSc from a different species as substrate is compatible with the idea that the strain of origin, such as BSE, might comprise a molecular cloud of conformers. When a prion strain is introduced into the target or intermediate species, a subset of conformers is selected for amplification (Collinge and Clarke, 2007; Krejciova et al., 2014; Collinge, 2016). In this model, if a large subset of conformers of the original prion strain can replicate in the target species, the species barrier will be low. The size of the subset of conformers that can replicate in both species will partly be determined by the primary structure of PrP from both species.

An alternative but not mutually exclusive model is described as “deformed templating.” In this model, when a prion strain is introduced into a new species, PrP primary structural differences between the species impose molecular constraints that force the generation of a range of new PrPSc variants (Makarava and Baskakov, 2012; Makarava et al., 2013;
FIGURE 3 | Investigating inter-species transmissibility by PMCA and saPMCA. The potential for a prion strain to cross the species barrier between the species of origin (dark green) and the target species (brown) can be assessed by a single round PMCA experiment. Alternatively, in serial automated PMCA (saPMCA), the amplification phase can be extended by multiple rounds in which the seed from one round is diluted into fresh PrP\textsuperscript{C} substrate. Multiple rounds of saPMCA using PrP\textsuperscript{C} from the species of origin might stabilize a prion strain to increase inter-species transmissibility. Alternatively, saPMCA with target species PrP\textsuperscript{C} could be used to examine the potential for a strain to adapt to a new replication environment. Finally, saPMCA can be used to address whether propagation with PrP\textsuperscript{C} from an intermediate species (light green) facilitates transmission to the target species.

Baskakov, 2014). One of these variants may prove to be particularly adept at replication in the new host, resulting in the emergence of a new strain. With either model, PMCA and saPMCA are capable of modeling strain adaptation, regardless of the mechanism.

The conformational selection or deformed templating models are consistent with the findings of Huor et al. (2019) who used both animal transmission studies and saPMCA to show evidence of a C-type BSE-like signature emerging from atypical scrapie. When atypical scrapie PrP\textsuperscript{Sc} seeds were amplified over several rounds of saPMCA using TgBov substrate, PrP\textsuperscript{Sc} with a BSE-like molecular profile was amplified, that could be clearly discriminated from scrapie-like PrP\textsuperscript{Sc} using PrP\textsuperscript{Sc} subtype specific antibodies. These results indicated the theoretical possibility that classic BSE might arise through cross-infection from atypical sheep scrapie. In this way PMCA can amplify subsets of these molecular clouds that point to the existence of minority strains that may exist within other strains. In this way, PMCA and saPMCA may offer insights into the provenance of certain prion strains.

**Zoonotic Threat After Strain Adaptation in an Intermediate Species**

An advantage of saPMCA is that amplification protocols can be concatenated, i.e., the end-product of one amplification protocol can be used to seed further amplification using substrate from an individual with a different genotype, or another species entirely (Figure 3). This approach is useful for investigating whether an animal prion disease becomes a threat to human health after cross-infection and propagation in an intermediate species. Humanized transgenic mouse brain (TgHu) expressing the three polymorphic genotypes of PRNP codon 129 (TgHuMM, TgHuMV, TgHuVV) have been commonly used as a PMCA substrate to examine the zoonotic threat posed by animal prion diseases (Barria et al., 2014b).
The species-specificity of certain anti-PrP antibodies has been used to evaluate the effect of in vitro studies of zoonotic potential. These antibodies specifically detect the conversion of the substrate PrP<sub>C</sub> to protease resistant PrP<sub>S</sub> without interference from the small amount of PrP<sub>C</sub> that was added as seed. For example, 3F4 recognizes the 106-112 epitope in human, but not ovine or bovine PrP.

Krejcirova et al. (2014) used 3F4 to show that C-type BSE from cattle or experimentally infected sheep could induce the conversion of human PrP<sub>C</sub> to PrP<sub>S</sub> in single-round PMCA. BSE PrP<sub>S</sub> that had retained its BSE-like molecular signature after amplification using an ARQ/ARQ ovine substrate also retained its ability to convert human PrP<sub>C</sub>. In both cases, conversion was dependent on the codon 129 genotype of human PrP being MM. However, BSE PrP<sub>S</sub> that had adapted and acquired a scrapie-like molecular signature after amplification using VRQ/VRQ substrate had lost its ability to convert human PrP<sub>C</sub>. This experimental observation may suggest that BSE PrP<sub>S</sub> can adapt and adopt the guise of a scrapie-like strain in sheep, but this adaptation reduces its zoonotic threat to humans.

The effects of the adaption of BSE PrP<sub>S</sub> to sheep on its virulence toward humans have also been analyzed by animal transmission studies. Padilla et al., 2011 showed that BSE prions from cattle could infect TgHu mice that over-express PrP<sub>C</sub> MM at codon 129, albeit with a large transmission barrier. This barrier was significantly reduced when the same mouse models were challenged with PrP<sub>S</sub> from BSE-infected sheep and goats suggesting an increased zoonotic threat from BSE in these species (Padilla et al., 2011). However, it is not known what effect adaptation of BSE prions to sheep or goats by multiple passages would have on its virulence toward humans.

**Emergent and Atypical Animal Prion Diseases**

**Assessing the Zoonotic Threat of CWD and Atypical Forms of Scrapie and BSE**

The zoonotic threat to humans of BSE in cattle is well known. In contrast, there is no epidemiological evidence to support scrapie in sheep posing a risk to human health (Brown et al., 2003; van Duijn et al., 1998). The zoonotic threat of CWD in cervids, and atypical forms of both scrapie and BSE are unknown. However, a number of in vitro and transmission studies using TgHu mice or non-human primates suggest a significant barrier for CWD transmission to humans [see Table in Barria et al. (2014a) for a summary of the in vitro and in vivo approaches that have been used]. Furthermore, challenge of TgHu mice with atypical H- and L-type BSE, showed that L-type BSE is capable of transmitting disease to transgenic mice overexpressing a human PrP (Beringue et al., 2008).

Transmission studies using mice expressing human PrP, or non-human primates, provide a rigorous assessment of the zoonotic threat to humans posed by animal prion diseases (Brandner and Jaunmuktane, 2017). However, this approach is often slow and expensive. In contrast, in vitro conversion assays such as PMCA are fast and adaptable. They can be used to assess the potential for the heterologous conversion of human PrP by newly emerging animal prion diseases, or atypical forms of classic animal prion diseases. The approach of testing seeds from multiple species in parallel against substrate from one species (e.g., human) allows the zoonotic potential of several prion strains to be compared. The results of these experiments can be judged alongside evidence from epidemiology and transmission studies.

An interesting observation of many of these comparisons is the concept of conversion efficiency as a possible measure of zoonotic potential, which can be inferred from the levels of PrP<sub>S</sub> that have accumulated after a defined period of amplification. In this regard, the reliability of PMCA for modeling transmission barriers has been evaluated by Levavasseur et al. (2014). Using vCJD and atypical BSE seeds against various transgenic mouse brain substrates, a good agreement between the amplification factors obtained by PMCA results and attack rates for parallel in vivo transmission experiments was observed (Levavasseur et al., 2014).

Chronic wasting disease is an acquired prion disease of cervids that affects captive and free-ranging deer and elk populations primarily in North America but recently reported in Europe (Sigurdson and Aguzzi, 2007; Benestad et al., 2016). Recently, PMCA has been used to study strain adaptation of CWD prions to a range of cervid host PrP<sub>C</sub> sequences, suggesting there could be a constantly evolving diversity of CWD conformers. This has implications for the potential emergence of CWD in new Cervid species (Duque Velasquez et al., 2020) that may in turn pose a threat to human health.

Protein misfolding cyclic amplification has been used to assess the molecular compatibility and the direct zoonotic threat that CWD might pose to humans. Barria et al., 2011 reported that CWD PrP<sub>S</sub> from mule deer (Odocoileus hemionus) could convert human PrP from TgHu substrate to produce PrP<sub>S</sub> with a unique molecular profile, contrasting to the observations reported by Kurt et al. (2009, 2015) of a significant species barrier between humans and cervids; However, extensive in vitro conditioning of the mule deer CWD isolate (using cervid PrP<sub>C</sub> substrate in PMCA or serial transmission in a cervidised transgenic mouse model) was necessary to overcome the molecular barrier and support the conversion of the human PrP<sub>C</sub> (Kurt et al., 2009, 2015; Barria et al., 2011).

In a later study, Barria et al., 2014a used PMCA to address the possibility of direct zoonotic transmission of prions from cervids to humans, and compared the results with scrapie and BSE prions. Using PrP<sub>C</sub> substrate from human, TgHu mouse brain and 293F cultured cells, we showed that CWD PrP<sub>S</sub> from North American elk (Cervus canadensis) could convert PrP<sub>C</sub>, albeit less efficiently than BSE (Barria et al., 2014a). This study concluded that there were no absolute molecular barriers for CWD to infect humans.

In addition, the zoonotic potential of CWD was further examined by testing prion seeds from North American elk of different cervid PRNP codon 132 genotypes and wild-tailed deer (O. virginianus), against TgHu substrate in a single-round PMCA. All cervid seeds induced conversion of human PrP<sub>C</sub>, although conversion efficiency depended on the compatibility of
the codon 132 genotype of cervid PrP and the corresponding codon 129 of human PRNP (Barria et al., 2018b).

**Zoonotic Threat of Atypical Animal Prions**

Atypical scrapie, also known as Nor98, is a rare prion disease of sheep identified as a result of active surveillance for scrapie (Benestad et al., 2003). In PMCA investigations of its zoonotic potential, Nor98 failed to induce significant conversion using PrPSc substrate from human, TgHu mouse brain and 293 F cultured cells in contrast to BSE and human CJD positive controls (Barria et al., 2014a).

Atypical BSE is a rare, atypical prion disease of cattle comprising two forms: L-type BSE and H-type BSE. L-type BSE, also known as bovine amyloidotic spongiform encephalopathy, is neuropathologically characterized by amyloid plaques in the brain, and has a PrPSc subtype similar to type 2A in human sCJD patients, with a faster migrating unglycosylated fragment compared with C-type BSE (Casalone et al., 2004). The other atypical form, H-type BSE, is characterized by a slower migrating protease resistant PrPSc fragments compared with C-type BSE (Biacabe et al., 2004). In contrast to C-type BSE which is acquired, L-type and H-type BSE are believed to be sporadic.

Protein misfolding cyclic amplification was used to assess the potential for atypical BSE to cross the species barrier to humans. Neither L-type nor H-type BSE induced significant conversion in PMCA using PrPSc substrate from human, TgHu mouse brain and 293 F cultured cells (Barria et al., 2014a,b), in contrast to C-type BSE and human CJD positive controls (Barria et al., 2014a). This could be taken to suggest that the atypical forms of BSE pose a poor zoonotic threat, or are far less likely to transmit to humans compared to classic BSE. However, an earlier finding reported that L-type BSE was able to produce infectivity in a humanized transgenic mice model overexpressing the human prion protein (Beringue et al., 2008).

**Zoonotic Threat of CWD Emerging in Europe**

The first identification of CWD in Europe occurred in 2016 in wild moose (Aelos alces, also known as Eurasian elk) and in a free-ranging reindeer (Rangifer tarandus, closely related to the free-ranging caribou of North America), a species not previously known to be affected by CWD in wild or farmed animals (Benestad et al., 2016). The molecular compatibility and zoonotic potential of CWD in reindeer was examined by testing prion seeds from two reindeer experimentally infected with CWD from white-tailed deer (Mitchell et al., 2012). Seeds prepared from these reindeer brains were tested against TgHuMM, TgHuMY, TgHuVV substrates in a single-round PMCA (Barria et al., 2018b). This study again used the 3F4 monoclonal antibody to detect the induced conversion of human PrPSc to protease resistant PrPSc. Samples of PrPSc from one of the two CWD infected reindeer could induce robust conversion of MM, MV, andVV substrates. The other reindeer had a lower abundance of PrPSc and showed weaker conversion of all three substrate genotypes tested. Barria et al., 2018b showed that low levels of experimentally infected reindeer brain PrPSc could be partially purified and normalized (according to the level of protease-resistant PrPSc) and used to successfully seed PMCA reactions. The influence of codon 129 on the molecular barriers for conversion of human PrPSc by reindeer PrPSc is currently being further investigated. Consistent with our previous findings and those reported previously by Raymond et al. (1997, 2000) and (Barria et al., 2011, 2014a,b, 2018b), we concluded that there were no absolute molecular barriers to the conversion of human PrPSc by CWD PrPSc, and a more comprehensive and thorough assessment of the zoonotic potential of CWD might be needed.

**Investigating the Potential of CWD to Infect Other Non-human Species**

The above PMCA studies involved comparing seeds from multiple species against substrate from one species, namely humans. An alternative approach is to test one seed on substrates from multiple species. For example, this approach can be used to assess the threat posed by CWD infected cervids to other species that share its environment in the wild. Furthermore, PrP sequence alignment can be used to assess the key determinants of the species barrier. Kurt et al., 2009 used saPMCA to show that vole and field mouse PrPSc supported serial amplification of white tailed deer (WTD) CWD PrPSc, whereas prairie dog and coyote PrPSc did not. Sequence comparisons of multiple species substrates showed that asparagine at codon 170 of the substrate PrPSc was important for supporting trans-species conversion (Kurt et al., 2009).

The potential for cross transmission of prion disease between cervids and sheep has been investigated using another version of the PMCA concept, namely recPMCA (Figure 4). This method is a variation of saPMCA that focuses on the effect of PrP sequence differences on molecular compatibility (Erana et al., 2017; Fernandez-Borges et al., 2017). When recPMCA is used to investigate species barriers, the seeds from the species of origin are firstly amplified in multiple rounds of PMCA using bacterially expressed recombinant PrP (recPrP) of the same species. Although unglycosylated, it is assumed that the recPrPSc end products retain some of the conformational properties of the original seed. The levels of the recPrPSc seeds are normalized, before being serial diluted and used to seed PMCA reactions containing wild-type or mutant recPrP from one or more target species (Erana et al., 2017; Harrathi et al., 2019). The advantage of using recPrP as a substrate is that mutant forms can be readily engineered to investigate the effects of amino acid substitutions, deletions, or insertions.

Using recPMCA Harrathi et al. (2019) were unable to serially convert wild-type sheep recPrP substrate using CWD recPrPSc, indicating a significant species barrier for the transmission of CWD to sheep. However, single residue substitutions at four sites in ovine PrP affected its potential to be converted by CWD recPrPSc, which offered insights into PrP sequence-structure factors affecting the potential for cross-infection from cervids to sheep, such as the β2-α2 loop (Harrathi et al., 2019). It should be noted that transmission studies where sheep have been challenged with mule deer CWD, and elk challenged with sheep scrapie, suggest that the species barrier
FIGURE 4 | Recombinant PMCA (recPMCA). In recPMCA, bacterially expressed and purified recombinant PrP (recPrP) is used as a substrate for conversion. Firstly, PrP<sup>Sc</sup> from infected tissue of the species of origin (dark green) is propagated by saPMCA using recPrP from the same species to produce recPrP<sup>Sc</sup> as an end product, detectable on western blots as a single band. Propagation in recPMCA requires the presence of “helper” brain homogenate from PRNP<sup>–/–</sup> animals. Thus, the effects of variable PrP<sup>C</sup> expression, glycosylation and cofactors that affect conventional PMCA reactions are largely eliminated, to focus primarily on the effect of sequence and conformational compatibility. The levels of recPrP<sup>Sc</sup> are normalized and the recPrP<sup>Sc</sup> is serially diluted in recPrP from the target species before being subjected to cycles of sonication and incubation. Species barriers are assessed according to how abruptly PMCA conversion is diminished by serial dilution of the seed: i.e., if the barrier is low, highly diluted seed will still cause conversion. Mutant forms of recPrP can easily be generated to examine the effect of amino acid substitutions.

Advantages and Limitations of saPMCA for Assessing Zoonotic Potential

For prion diseases, some of the factors linked to the species barrier phenomenon include (1) differences between the PRNP sequences of the species (2) the animal prion strain, as enciphered in the conformation of PrP<sup>Sc</sup>, and (3) the physiological differences between humans and the animal in question. The effect of cofactors on prion conversion and the glycosylation of PrP<sup>C</sup> are two other species-specific phenomena that contribute to the species barrier. PMCA can model aspects associated with the molecular and subcellular compatibility of the PrP<sup>Sc</sup> structure and PrP sequence, previously referred to as the “sequence – structure barrier” (Baskakov, 2014). Although PMCA may be limited in its ability to model additional factors operating above the subcellular level, it provides a powerful method for investigating the features affecting the molecular compatibility between heterologous PrP<sup>Sc</sup> and PrP<sup>C</sup> that are still not fully understood. The fact that PMCA produces bona fide PrP<sup>Sc</sup> as an end product means that PMCA can help to indicate the molecular profile that may arise from a cross-species infection. The properties of the PrP<sup>Sc</sup> end product can be assessed, such as the western blot profile, or its equilibrium dissociation curve following denaturation with increasing concentrations of guanidine HCl (Yokoyama et al., 2011). Furthermore, this product can be used in onward animal transmission studies, to obtain additional strain characteristics including clinical signs, the neuroanatomical distribution of lesions and PrP<sup>Sc</sup> deposition and incubation periods (Beck et al., 2013).
A practical advantage of PMCA for modeling species barriers is speed. A single round of PMCA typically takes 48 h, although multiple rounds of saPMCA may take several weeks. However, this is considerably shorter than the months or years taken to complete animal transmission studies.

An important consideration in PMCA is ensuring the availability and consistency of the substrate. Conversion is most efficient and reliable using perfused brain from experimental animals, usually transgenic mice. For assessing the zoonotic threat to humans, post-mortem human brain tissue with consent for use can be used, but there are factors that confound conversion efficiency and consistency, including post mortem interval, the presence of blood, and variability between samples in terms of the relative content of gray and white matter. To improve consistency, the use of a stably transfected human cell cultures as a source of human PrP Sc substrate for PMCA has been demonstrated (Yokoyama et al., 2011; Barria et al., 2014a,b).

The drive toward more efficient PMCA can have its own counterpoint. Some particular observations suggest we should be cautious when modeling species or genotypic barriers with PMCA. For instance, Vidal et al. (2013) were able to amplify BSE PrP Sc by saPMCA using PrP C from rabbits and dogs, even though the latter species are considered resistant to prion disease. Lacroux et al. (2014) have developed a PMCA assay using ovine Q171 substrate which can efficiently amplify BSE/vCJD prions regardless of the species of origin. Therefore, with their methodology, PrP sequence homology between seed and substrate does not appear to be crucial for BSE/vCJD prion replication in vitro (Lacroux et al., 2014). It has also been shown that unseeded saPMCA using leporid PrP Sc substrate could lead to the de novo formation of PrP Sc, which could induce disease when used to inoculate rabbits (Chianini et al., 2012). This suggests that increases in the efficiency of PMCA can circumvent species barriers, and induce disease in species that were thought to be resistant, and therefore careful interpretation of the results of PMCA experiments is required.

Even when a prion strain is known to be zoonotic, PMCA could potentially underestimate the species barrier. For instance, several PMCA studies have shown that human PrP C (M at codon 129) is readily converted by BSE PrP Sc in a manner dependent upon the genotype at PRNP codon 129 (Raymond et al., 1997; Jones et al., 2009; Barria et al., 2014a; Krecjiova et al., 2014). However, animal transmission studies pointed to a higher transmission barrier for BSE between cattle and humans. Transmission of cattle BSE to TgHu mice overexpressing human PrP C has been shown to be inefficient (Asante et al., 2002; Padilla et al., 2011), and no transmission of cattle BSE was observed using an alternative gene-targeted TgHu mouse model (Bishop et al., 2006). It should be noted that the extent of the vCJD epidemic was relatively small compared with the very high numbers cattle that were infected with BSE. Therefore, the complementation of in vitro and in vivo models are important to understand the complexity surrounding species barrier and the zoonotic potential to humans.

**CONCLUSION**

Where as animal transmission studies are an effective means for investigating species barriers, PMCA continues to provide a time- and cost-efficient alternative that can offer genuine insights into the molecular feasibility of cross-species transmission, and some of the strain properties of PrP Sc that may arise. PMCA can be used to assess whether prion strains can transcend species barriers, and also genotypic barriers within the same species. Unlike other in vitro conversion assays, PMCA can predict alterations in the molecular profile of PrP Sc that might occur upon adaptation to a new species. PMCA can also uncover evidence of traceback, a give indications on the likely origin of a prion strain. Also, PMCA, using substrate from the target species, can be used to model the molecular compatibility and zoonotic risk of the prion agent. In summary, the PMCA group of techniques provide a powerful approach for assessing intra- and inter-species barriers, focused on the molecular feasibility of prion conversion. However, the results should be interpreted with caution, and can be complemented with animal transmission and epidemiological studies in circumstances where they are available.

**AUTHOR CONTRIBUTIONS**

AP, SS, and MB: writing—original draft preparation, review and editing. MB: conceptualization. All authors: read and agreed to the published version of the manuscript.

**FUNDING**

This report is independent research commissioned and funded by the Department of Health and Social Care Policy Research Programme and the Government of Scotland [“The National CJD Research and Surveillance Unit (NCJDRSU),” PR-ST-0614-00008_18].

**ACKNOWLEDGMENTS**

The authors wish to thank Fraser Brydon for his valuable time in proofreading this review. All figures were created with BioRender package.

**REFERENCES**

Asante, E. A., Linehan, J. M., Desbruslais, M., Joiner, S., Gowland, I., Wood, A. L., et al. (2002). BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *Embo J.* 21, 6358–6366. doi: 10.1093/emboj/ cdf653

Babelhadj, B., Di Bari, M. A., Pirisinu, L., Chiappini, B., Gaouar, S. B. S., Riccardi, G., et al. (2018). Prion disease in dromedary camels, Algeria. *Emerg. Infect. Dis.* 24, 1029–1036. doi: 10.3201/eid2406.172007
Barria, M. A., Balachandran, A., Morita, K., Kitamoto, T., Barron, R., Manson, J., et al. (2014a). Molecular barriers to zoonotic transmission of prions. Emerg. Infect. Dis. 20, 88–97. doi: 10.3201/eid2001.130858

Barria, M. A., Ironside, J. W., and Head, M. W. (2014b). Exploring the zoonotic potential of animal prion diseases: in vivo and in vitro approaches. Prion 8, 85–91. doi: 10.4161/prion.28124

Barria, M. A., Lee, A., Green, A. I., Knight, R., and Head, M. W. (2018a). Rapid amplification of prions from variant Creutzfeldt-Jakob disease cerebrospinal fluid. J. Pathol. Clin. Res. 4, 86–92. doi: 10.1002/cjpr.2090

Barria, M. A., Libori, A., Mitchell, G., and Head, M. W. (2018b). Susceptibility of human prion protein to conversion by chronic wasting disease Prions. Emerg. Infect. Dis. 24, 1482–1489. doi: 10.3201/eid2408.161888

Barria, M. A., Telling, G. C., Gambetti, P., Mastriani, J. A., and Soto, C. (2011). Generation of a new form of human PrP(Sc) in vitro by interspecies transmission from cervid prions. J. Biol. Chem. 286, 7490–7495. doi: 10.1074/jbc.m110.198465

Baskakov, I. V. (2014). The many shades of prion strain adaptation. Prion 8, 169–172. doi: 10.4161/17504836

Beck, K. E., Thorne, L., Lockey, R., Vickery, C. M., Terry, L. A., Bujdoso, R., et al. (2014). Transmission of scrapie to rhesus monkeys by oral feeding of scrapie to sheep. Emerg. Infect. Dis. 20, 901–907. doi: 10.3201/eid2006.140808

Brandner, S., and Jaunmuktane, Z. (2017). Prion disease: experimental models and reality. Acta Neuropathol. 133, 197–222. doi: 10.1007/s00401-017-1670-5

Brown, P. (1998). Transmission of spongiform encephalopathy through biological products. Dev. Biol. Stand. 93, 73–78.

Brown, P., Brandel, J. P., Sato, T., Nakamura, Y., MacKenzie, J., Will, R. G., et al. (2012). Iatrogenic Creutzfeldt-Jakob disease, final assessment. Emerg. Infect. Dis. 18, 901–907.

Brown, P., Cathala, F., Raubertas, R. F., Gajdusek, D. C., and Castaigne, P. (1987). The epidemiology of Creutzfeldt-Jakob disease: conclusion of a 15-year investigation in France and review of the world literature. Neurology 37, 895–904. doi: 10.1212/wnl.37.6.895

Bruce, M. E., Will, R. G., Ironside, J. W., McConnell, I., Drummond, D., Sutcliffe, A., et al. (1997). Transmissions to mice indicate that ‘new variant’ CJD is caused by the BSE agent. Nature 389, 498–501. doi: 10.1038/359557

Cali, I., Castellani, R., Alshekhelee, A., Cohen, Y., Blevins, J., Yuan, J., et al. (2009). Co-existence of scrapie prion protein types 1 and 2 in sporadic Creutzfeldt-Jakob disease: its effect on the phenotype and prion-type characteristics. Brain 132, 2643–2658. doi: 10.1093/brain/awp196

Cali, I., Lavrích, J., Moda, F., Kofsky, D., Nemaní, S. K., Appleby, B., et al. (2019). PMCA-replicated PrP(Sc) in urine of vCJD patients maintains infectivity and strain characteristics of brain PrP(PrD): transmission study. Sci. Rep. 9:5191.

Casalone, C., Zanusso, G., Acutia, P., Ferrari, S., Capucci, L., Tagliavini, F., et al. (2004). Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc. Natl. Acad. Sci. U.S.A. 101, 3065–3070. doi: 10.1073/pnas.0305777101

Cassmann, E. D., Frese, R. D., and Greenlee, J. J. (2021). Second passage of chronic wasting disease of mule deer to sheep by intracranial inoculation compared to classical scrapie. J. Vet. Diagn. Invest. 33, 711–720. doi: 10.1177/1040638721107615

Castilla, J., Gonzalez-Romero, D., Saá, P., Morales, R., De Castro, J., and Soto, C. (2008a). Crossing the species barrier by PrPSc replication in vitro generates unique infectious prions. Cell 134, 757–768. doi: 10.1016/j.cell.2008.07.030

Castilla, J., Morales, R., Saa, P., Barria, M., Gambetti, P., and Soto, C. (2008b). Cell-free propagation of prion strains. EMBO J. 27, 2557–2566. doi: 10.1038/emboj.2008.181

Castilla, J., Saá, P., Hetz, C., and Soto, C. (2005). In vitro generation of infectious scrapie cells. Cell 121, 195–206. doi: 10.1016/j.cell.2005.02.011

Castilla, J., Saá, P., Morales, R., Abid, K., Maundrell, K., and Soto, C. (2006). Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Methods Enzymol. 412, 3–21. doi: 10.1016/S0076-6879(06)41201-1

Chianini, F., Fernandez-Borges, N., Vidal, E., Gibbard, L., Pintado, B., De Castro, J., et al. (2012). Rabbits are not resistant to prion infection. Proc. Natl. Acad. Sci. U.S.A. 109, 5080–5085.

Collinge, J. (2016). Mammalian prions and their wider relevance in neurodegenerative diseases. Nature 539, 217–226. doi: 10.1038/nature2415

Collinge, J., and Clarke, A. R. (2007). A general model of prion strains and their pathogenicity. Science 318, 930–936. doi: 10.1126/science.1138718

Concha-Mariambio, L., Chacon, M. A., and Soto, C. (2020). Preclinical detection of prions in blood of nonhuman primates infected with variant Creutzfeldt-Jakob disease. Emerg. Infect. Dis. 26, 34–43. doi: 10.3201/eid2601.181422

Concha-Mariambio, L., Prizitzk N., Soto, C., Tagliavini, F., Ironside, J. W., Schulz, P. E., et al. (2016). Detection of prions in blood from patients with variant Creutzfeldt-Jakob disease. Sci. Transl. Med. 8:370ra183.

Delisle, M. B., Fabre, N., Rochiccioli, P., Doerr-Schott, J., Rumeau, J. L., and Bes, A. (1993). [Creutzfeldt-Jakob disease after treatment with human extracted growth hormone. A clinicopathological study]. Rev. Neurol. (Paris) 149, 524–527.

Deslys, J. P., Jaegly, A., d’Aignaux, J. H., Mouthon, F., de Villemeur, T. B., and Dormont, D. (1998). Genotype at codon 129 and susceptibility to Creutzfeldt-Jakob disease. Lancet 351:1251. doi: 10.1016/s0140-6736(97)9317-x

Duffy, P., Wolf, J., Collins, G., DeVoe, A. G., Streeten, B., and Cowen, D. (1974). Letter: possible person-to-person transmission of Creutzfeldt-Jakob disease. N. Engl. J. Med. 290, 692–693. doi: 10.1056/NEJM1974032029012

Duque Velasquez, C., Kim, C., Haldeman, T., Kim, C., Herbst, A., Aiken, J., et al. (2020). Chronic wasting disease (CWD) prion strains evolve via adaptive diversification of conformers in hosts expressing prion protein polymorphisms. J. Biol. Chem. 295, 4985–5001. doi: 10.1074/jbc.r2010254

Erana, H., Fernandez-Borges, N., Elezgarai, S. R., Harrathi, C., Charco, J. M., Chianini, F., et al. (2007). Sheep-passaged bovine spongiform encephalopathy agent exhibits altered pathobiological properties in bovine-PrP transgenic mice. J. Virol. 81, 835–843. doi: 10.1128/jvi.01356-06
Fernandez-Borges, N., Erana, H., Elezgarai, S. R., Harrath, C., Venegas, V., and Castilla, J. (2017). A quick method to evaluate the effect of the amino acid sequence in the misfolding promenance of the Prion protein. Methods Mol. Biol. 1658, 205–216. doi: 10.1007/978-1-4939-7244-9_15

Foster, J. D., Parnham, D., Chong, A., Goldmann, W., and Hunter, N. (2001). Clinical signs, histopathology and genetics of experimental transmission of BSE and natural scrapie to sheep and goats. Vet. Rec. 148, 165–171. doi: 10.1136/vr.148.6.165

Gibbs, C. J. Jr., Joy, A., Heffner, R., Franko, M., Miyazaki, M., Asher, D. M., et al. (1985). Clinical and pathological features and laboratory confirmation of Creutzfeldt-Jakob disease in a recipient of pituitary-derived human growth hormone. N. Engl. J. Med. 313, 734–738. doi: 10.1056/nejm198509193131207

Goldmann, W. (2008). PrP genetics in ruminant transmissible spongiform encephalopathies. Vet. Res. 39:30. doi: 10.1051/vetres:2008010

Gonzalez, L., Martin, S., and Jeffrey, M. (2003). Distinct profiles of PrP(d) immunoreactivity in the brain of scrapie- and BSE-infected sheep: implications for differential cell targeting and PrP processing. J. Gen. Virol. 84, 1339–1350. doi: 10.1099/vir.0.18800-0

Green, K. M., Castilla, J., Seward, T. S., Napier, D. L., Jewell, E. J., Soto, C., et al. (2008). Accelerated high fidelity prion amplification within and across prion species barriers. PLoS Pathog. 4:e1000139. doi: 10.1371/journal.ppat.1000139

Greenlee, J. I. (2019). Review: update on classical and atypical scrapie in sheep and goats. Vet. Pathol. 56, 6–16. doi: 10.1177/030098581984247

Griffith, J. S. (1967). Self-replication and scrapie. Nature 215, 1043–1044. doi: 10.1038/2151043a0

Halliez, S., Passet, B., Martin-Lannerée, S., Hernandez-Rapp, J., Laude, H., Mouillet-Richard, S., et al. (2014). To develop with or without the prion protein. Front. Cell Dev. Biol. 2:58. doi: 10.3389/fcell.2014.00058

Hamir, A. N., Miller, J. M., Cullip, R. C., Kunke, R. A., Jenny, A. L., Stud, M. I., et al. (2004). Transmission of sheep scrapie to elk (Cervus elaphus nelsonii) by intracerebral inoculation: final outcome of the experiment. J. Vet. Diagn. Invest. 16, 316–321. doi: 10.1177/104063870401600410

Harrath, C., Fernandez-Borges, N., Erana, H., Elezgarai, S. R., Venegas, V., Charco, J. M., et al. (2019). Insights into the bidirectional properties of the sheep-deer prion transmission barrier. Mol. Neurobiol. 56, 5287–5303. doi: 10.1007/s12035-019-2443-8

Head, M. W., and Ironside, J. W. (2012). Review: creutzfeldt-jakob disease: prion protein type, disease phenotype and agent strain. Neuropathol. Appl. Neurobiol. 38, 296–310. doi: 10.1111/j.1365-2990.2012.01265.x

Hill, A. F., Desbruslais, M., Joiner, S., Siddle, K. C., Gowland, L., Collinge, J., et al. (1997). The same prion strain causes vCJD and BSE. Nature 389, 488–490. 526. doi: 10.1038/3892852

Hope, J. (2013). Bovine spongiform encephalopathy: a tipping point in One Health and Food Safety. Curr. Top. Microbiol. Immunol. 366, 37–47. doi: 10.1007/978-3-662-45791-7_264

Hunter, N., and Bossers, A. (2006). Prions in Humans and Animals, eds B. Hörnlimann, D. Riesner, H. Kretzschmar (Berlin: de Gruyter), 640–647.

Hvr, A., Espinosa, J. C., Vidal, E., Cassard, H., Douet, J. Y., Lugan, S., et al. (2021). Prion strains and transmission barrier Phenomena. PLoS Pathogens 10:e1004202. doi: 10.1371/journal.ppat.1004202

Larouche, C., Comoy, E., Moutoud, M., Perret-Liaudet, A., Lugan, S., Litaute, C., et al. (2014). Preclinical detection of variant CJD and BSE prions in blood. PLoS Pathogens 10:e1004202. doi: 10.1371/journal.ppat.1004202

Levavasseur, E., Privat, N., Martin, J. C. E., Simonneau, S., Baron, T., Flan, B., et al. (2014). Molecular modeling of prion transmission to humans. Virus Res. 6, 3766–3777. doi: 10.3390/v6103766

Lov, S., Budka, H., Ironside, J. W., and Perry, A. (2015). Greenfield’s Neuropathology Vols 1 and 2, Volume 2: VII-VIII’, 9th Edn, Vol. 2. Boca Raton, London, New York: CRC Press, Taylor and Francis Group.

Makarava, N., and Baskakov, I. V. (2012). Genes of transmissible protein states via deformed templating. Prion 6, 252–255. doi: 10.4116/pr.19930

Makarava, N., Svatchenko, R., and Baskakov, I. V. (2013). Selective amplification of classical and atypical prions using modified protein misfolding cyclic amplification. J. Biol. Chem. 288, 33–41. doi: 10.1074/jbc.M112.419531

Mitchell, G. B., Sigurdson, C. J., O’Rourke, K. I., Algire, J., Harrington, N. P., Walther, I., et al. (2012). Experimental oral transmission of chronic wasting disease PrPSc. J. Neurol. 213, 21–26. doi: 10.1002/jpd.2204

Mody, F., Gambetti, P., Notari, S., Concha-Marambio, L., Catania, M., Park, K. W., et al. (2014). Prions in the urine of patients with variant creutzfeldt-jakob disease. N. Engl. J. Med. 371, 530–539.

Mody, F., Suardi, S., Di, Fede G., Indaco, A., Lirmido, L., Vincenzi, C, et al. (2012). MM2-thalamic Creutzfeldt-Jakob disease: neuropathological, biochemical and
transmission studies identify a distinctive prion strain. *Brain Pathol.* 22, 662–669. doi: 10.1111/j.1750-3639.2012.00572.x

Mok, T., Jaunmuktane, Z., Joiner, S., Campbell, T., Morgan, C., Wakerley, B., et al. (2017). Variant Creutzfeldt-Jakob Disease in a Patient with Heterozygosity at PRNP Codon 129. *N. Engl. J. Med.* 376, 292–294. doi: 10.1056/nejmc1616003

Padilla, D., Berezynska, V., Espinosa, J. C., Andreotti, O., Jaunmuktane, E., Reine, F., et al. (2011). Sheep and goat BSE propagate more efficiently than cattle BSE in human PrP transgenic mice. *PLoS Pathog.* 7:e1001319. doi: 10.1371/journal.ppat.1001319

Palmer, M. S., Dryden, A. J., Hughes, J. T., and Collinge, J. (1991). Homozygous human transmissible spongiform encephalopathies. *J. Clin. Microbiol.* 40, 972–980. doi: 10.1128/jcm.40.3.972-980.2004

Parchi, P., Giese, A., Capellari, S., Brown, P., Schulz-Schaeffer, W., Windl, O., et al. (2000). Genetic influence on the structural variations of the abnormal prion protein. *Brain* 127, 2348–2359. doi: 10.1093/brain/awh2249

Polymenidou, M., Stoeck, K., Glattel, M., Vey, M., Bellon, A., and Aguzzi, A. (2005). Coexistence of multiple PrPSc types in individuals with Creutzfeldt-Jakob disease. *Lancet Neurol.* 4, 805–814. doi: 10.1016/s1474-4422(05)70225-8

Powell-Jackson, J., Weller, R. O., Kennedy, P., Creeke, M. A., Whitcomb, E. M., and Newcom-Davis, J. (1983). Creutzfeldt-Jakob disease after administration of human growth hormone. *Lancet* 2, 244–246. doi: 10.1016/s0140-6736(85)90292-2

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science* 216, 136–144. doi: 10.1126/science.6801762

Raymond, G. J., Bosser, A., Raymond, L. D., O’Rourke, K. I., McHolland, L. E., Bryant, P. K. 3rd, et al. (2000). Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. *Embo J.* 19, 4425–4430. doi: 10.1038/smb19.17.4425

Raymond, G. J., Hope, J., Kocsisko, D. A., Prisold, S. A., Raymond, L. D., Bosser, A., et al. (1997). Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature* 388, 285–288. doi: 10.1038/40876

Richt, J. A., and Hall, S. M. (2008). BSE case associated with prion protein gene mutation. *PLoS Pathog.* 4:e1000156. doi: 10.1371/journal.ppat.1000156

Ritchie, D. L., and Barria, M. A. (2021). Prion diseases: a unique transmissible agent or a model for neurodegenerative diseases? *Biomolecules* 11:207. doi: 10.3390/biom11020207

Ritchie, D. L., Barria, M. A., Peden, A. H., Yuil, H. M., Kirkpatrick, J., Adlard, P., et al. (2017). UK iatrogenic Creutzfeldt-Jakob disease: investigating human prion transmission across genotypic barriers using human tissue-based and molecular approaches. *Acta Neuropathol.* 133, 579–595. doi: 10.1007/s00401-016-1838-x

Rudge, P., Jaunmuktane, Z., Adlard, P., Bjurstrom, N., Caine, D., Lowe, J., et al. (2015). Iatrogenic CJD due to pituitary-derived growth hormone with genetically determined incubation times of up to 40 years. *Brain* 138, 3386–3399. doi: 10.1093/brain/aws235

Sabirio, G. P., Permanne, B., and Soto, C. (2001). Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* 411, 810–813. doi: 10.1038/35081105

Sigurdson, C. J., and Aguzzi, A. (2007). Chronic wasting disease. *Biochim. Biophys. Acta* 1772, 610–618

Stack, M., Gonzalez, L., Jeffrey, M., Martin, S., Macaldowie, C., Chaplin, M., et al. (2009). Three serial passages of bovine spongiform encephalopathy in sheep do not significantly affect discriminatory test results. *J. Gen. Virol.* 90, 764–768. doi: 10.1099/vir.0.005983-0

Stack, M., Jeffrey, M., Gubbins, S., Grimmer, S., Gonzalez, L., Martin, S., et al. (2006). Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998-2004. *J. Gen. Virol.* 87, 2099–2107. doi: 10.1099/vir.0.81254-0

Takeuchi, A., Kobayashi, A., Parchi, P., Yamada, M., Morita, M., Uno, S., et al. (2016). Distinctive properties of plaque-type dura mater grafted-associates Creutzfeldt-Jakob disease in cell-protein misfolding cyclic amplification. *Laboratory Invest.* 96, 581–587. doi: 10.1038/labinvest.2016.27

Thuring, C. M., Erkens, J. H., Jacobs, J. G., Bosser, A., Van Keulen, L. J., Garsen, G. J., et al. (2004). Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. *J. Clin. Microbiol.* 42, 972–980. doi: 10.1128/jcm.42.3.972-980.2004

Uro-Coste, E., Cassard, H., Simon, S., Lugan, S., Bilheude, J. M., Perret-Liaudet, A., et al. (2008). Beyond PrPProtease type 1/type 2 dichotomy in Creutzfeldt-Jakob disease. *PLoS Pathog.* 4:e1000029. doi: 10.1371/journal.ppat.1000029

Urwin, P. J., Mackenzie, J. M., Llewellyn, C. A., Will, R. G., and Hewitt, P. E. (2016). Creutzfeldt-Jakob disease and blood transfusion: updated results of the UK Transfusion Medicine Epidemiology Review Study. *Vox. Sang* 110, 310–316. doi: 10.1111/vox.12371

Vidal, E., Fernandez-Borges, N., Pintado, B., Ordonez, M., Marquez, M., Fendevilla, D., et al. (2013). Exploring the risks of a putative transmission of BSE to new species. *Prion* 7, 443–446. doi: 10.4161/prion.27014

Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., et al. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet* 347, 921–925.

Will, R. G., and Matthews, W. B. (1982). Evidence for case-to-case transmission of Creutzfeldt-Jakob disease. *J. Neurol. Neurosurg. Psychiatry* 45, 235–238. doi: 10.1136/jnnp.45.2.235

Wulf, M., A. A., and Aguzzi, A. (2017). The biological function of the cellular prion protein: an update. *J. Biol. Chem.* 15:34. doi: 10.1186/s12915-017-0375-5

Yokoyama, T., Takeuchi, A., Yamamoto, M., Kitamoto, T., Ironside, J. W., and Morita, M. (2011). Heparin enhances the cell-protein misfolding cyclic amplification efficiency of variant Creutzfeldt-Jakob disease. *Neurosci. Lett.* 498, 119–123. doi: 10.1016/j.neulet.2011.04.072

Author Disclaimer: The views expressed in this publication are those of the author(s) and not necessarily those of the Department of Health and Social Care or the Government of Scotland.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Peden, Saleiman and Barria. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.