Developing early diagnostics for prion diseases

Graham S Jackson¹, Simon Mead^{1,2} & John Collinge^{*1,2}

Practice Points

- Current prevalence estimates for variant Creutzfeldt-Jakob disease indicate around one infection among 2000 UK capita.
- Prion disease is typically only diagnosed once patients are in an advanced stage of neurological decline.
- Assays for the early diagnosis of prion disease or screening applications require a high degree of specificity, which effectively limits a biochemical marker of infection to abnormal forms of the prion protein.
- The self-replicating nature of prions can be exploited for the detection of abnormalities in peripheral tissues, cerebrospinal fluid and blood *in vitro*.
- A blood test for variant Creutzfeldt-Jakob disease is currently being evaluated in clinical use.
- Quantitative progression markers of the disease will be required for clinical trials.

SUMMARY The diagnosis of prion disease is typically made late in the clinical course, by which time patients are in an advanced state of neurological decline. This is despite the presence of pathology in many tissues, particularly those of the lymphoreticular and central nervous systems. The recent description of an effective blood assay for variant Creutzfeldt-Jakob disease clearly demonstrates the potential for routine pre-mortem diagnosis, although further progress is required for the detection of sporadic forms of the disease.

Prion diseases are a group of rare, untreatable and transmissible neurodegenerative conditions of humans and animals that are caused by the misfolding of prion protein (PrP) [1,2]. Transmissibility of disease between humans and animals is a defining feature of the group, and has resulted in several high profile epidemics of acquired prion disease, including, for example, kuru in humans and bovine spongiform encephalopathy (BSE)

and its human counterpart variant Creutzfeldt-Jakob disease (vCJD). Prion diseases may also occur without any obvious exposure to an infectious source, the most common clinical syndrome being a rapidly progressive dementia known as sporadic Creutzfeldt-Jakob disease (CJD). Mutations in the *PRNP* gene cause autosomal, dominantly inherited prion disease. The infectious agent itself is largely or solely comprised

¹MRC Prion Unit, Department of Neurodegenerative Disease, University College London Institute of Neurology, Queen Square, London, WC1N 3BG, UK

²National Prion Clinic, National Hospital for Neurology & Neurosurgery, Queen Square, London, WC1N 3BG, UK

*Author for correspondence: Tel.: +44 207 837 4888; Fax: +44 207 837 8047; j.collinge@prion.ucl.ac.uk

of a misfolded and aggregated conformer of the normal cellular PrP, which may catalyze the conversion of further PrP to the disease-associated form by a templating process [3–5]. According to the prion model, PrP is absolutely central to the pathogenic process, since the key genetic risk factors for all prion diseases involve the *PRNP* gene itself [6]. Detection of abnormal forms of PrP is the main focus of strategies to develop new early diagnostics in human biofluids.

Human prion diseases & their importance for public health

In a typical population with a developed health-care system, approximately one to two individuals per million per year will be diagnosed with a prion disease. Approximately 85% of these patients will have sporadic CJD, which typically occurs with increasing incidence from late middle age upwards and has a uniform gender and geographical distribution. Sporadic CJD manifests clinically as a multifocal cortical dementia syndrome with additional neurological signs, typically cerebellar ataxia, myoclonus and motor signs (pyramidal and extrapyramidal). Rapid progression is the hallmark of sporadic CJD in most cases with a median survival of 4 months; although long-duration cases that persist for over 2 years are occasionally seen. Atypical presentations are well recognized in the early stages, regardless of presentation; however, most patients progress to a state of akinetic mutism prior to death. Unfortunately, approximately half of the patients are only diagnosed at this advanced premortem clinical stage. Inherited prion disease can have a similar clinical course to sporadic CJD; however, heterogeneity is a more marked feature and much more slowly progressive syndromes are commonly associated with certain specific gene mutations (historically termed the Gerstmann–Sträussler–Scheinker syndrome) [1].

The acquired prion diseases are less common and vary considerably between countries according to their respective population exposure to prions. vCJD has understandably provoked the greatest public health concerns. During the 1980s and early 1990s it is estimated that 2–3 million cattle infected with BSE from the cannibalistic recycling of animal carcasses in feed entered the human food chain in the UK [7,8]. The resulting transmission of the disease to humans was recognized after a succession of extremely young patients were diagnosed with

an atypical form of CJD, and subsequently over 200 individuals have died [9]. The early clinical features of vCJD are somewhat distinct from other forms of prion disease with a high prevalence of psychiatric symptoms, particularly low mood, and distal sensory disturbance, headache and chorea [10].

Several vCJD patients were active blood donors while incubating the disease and at an early stage in the outbreak there were understandable theoretical concerns that prions may be transmitted by blood transfusion. Public health concerns were heightened in 2003, 2006 and 2010 by the clinical transmission of vCJD by blood transfusion to three recipients who went on to die from the infection and evidence of asymptomatic infection in two further recipients (including one hemophiliac patient who had received vCJD-implicated fractionated blood products rather than red cell transfusion) [11–14]. Unlike sporadic CJD, vCJD prions are found in peripheral lymphoreticular tissues [15], a phenomenon that has allowed the anonymous screening of appendicular tissue [16]. These studies initially led to estimates of vCJD infection prevalence rates in the UK of approximately 1:4000. An extension of this study has resulted in a revision of these estimates upwards to 1:2000 (95% CI: 282–801 per million) [101]. At present, there is no way to exclude these individuals from donating blood. Recent animal work has further highlighted the potential importance of subclinical infection with vCJD, showing that, by measuring the rate of infection in peripheral tissues rather than clinical disease, the transmission of prions between species may be much more common [17]. Peripheral infection in vCJD also poses risk for surgical procedures as prions, which bind avidly to surgical steel, are relatively resistant to conventional sterilization techniques [18].

A more positive aspect of the transmissibility of prion disease is that this may be capitalized on in order to study the real disease in a laboratory setting, without recourse to animal models of uncertain validity. These studies have supported the testing of existing licensed drugs and rational screening for therapeutics [19]. Several agents have now been shown to delay the onset of clinical disease in prion-infected mice [20,21]. Most notably, monoclonal antibodies directed at the normal form of PrP are potent experimental therapeutics in a laboratory setting [22,23]. Small drug-like molecules and other macromolecules also show activity in this setting [24]. Clinical

trials have been carried out on two such examples, a nonopioid analgesic flupirtine and the antimalarial quinacrine; however, no evidence of clinical efficacy was shown [25,26]. In the absence of a validated biomarker of progression, or of therapeutic response, clinical trials have had to rely on crude milestones such as survival, which can be heavily influenced by several non-specific interventions for secondary infections. A consistent feature of laboratory studies is that compounds are more effective when given early in the incubation period of prion disease.

Diagnosis of human prion disease: current realities & the need for early diagnostic markers

The present realities of prion disease diagnosis in the UK are somewhat disappointing. Approximately half of sporadic CJD patients are diagnosed at an advanced stage of neurological decline, when they are immobile and unable to communicate [1]. It is hard to imagine a successful therapeutic intervention at this stage when, presumably, considerable neuronal loss has occurred and amounts of pathological PrP are substantial. Four key investigations are well established for use by neurologists: MRI of the brain, electroencephalography, cerebrospinal fluid (CSF) analysis for 14-3-3 protein and *PRNP* gene sequencing. MRI of the brain is a remarkably good diagnostic test, particularly if the imaging protocol includes diffusion-weighted imaging; considering abnormal signal change on fluid-attenuated inversion recovery, or diffusion-weighted imaging in the caudate, putamen, thalamus and cortex, sensitivities and specificity are over 90% based on the established clinical phase [27]. CSF testing is needed to exclude potentially treatable conditions such as encephalitis, and the presence of proteins indicative of rapid neuronal loss (14-3-3 and neuron-specific enolase) or gliosis (S100b) is supportive of nonspecific finding in the right clinical context [28]. Electroencephalography is historically important but is the least sensitive and specific of all routine diagnostics. *PRNP* gene sequencing is a direct definitive test for inherited prion disease and allows presymptomatic gene testing in at-risk family members following appropriate genetic counseling [29].

Despite the range of diagnostic tests available, early diagnosis is not achieved as often as it should be for a variety of reasons. The initial symptoms of prion diseases are often nonspecific in the relevant age categories (e.g., depression in young

people and cognitive problems in the elderly), the *a priori* chance of prion disease diagnosis at this nonspecific stage is low and it is usually not even considered. When rapid progression and obvious neurological clinical signs are noticed, flagging the undiagnosed condition as distinct from common psychiatric and neurodegenerative diseases, referrals are usually but not exclusively made to neurologists. At this stage, the understandable priority is the exclusion of treatable diagnoses such as limbic encephalitis caused by voltage-gated potassium channel antibodies, or primary CNS vasculitis, and therefore a wide panel of tests including the key prion disease diagnostics are ordered. The natural rapid history of prion disease and a sense of futility about treatment options result in diagnosis often being formalized weeks later, only a few days prior to death. Even in the absence of promising clinical trials, this reality of late diagnosis is a pity, as patients diagnosed early, prior to incapacitating cognitive dysfunction, have opportunities to organize the end of their life and make the best use of their remaining time without the distraction of further testing.

Abnormal PrP is a consistent, early & specific feature of prion disease

The intimate association between abnormal forms of PrP and prion disease pathology is long established and makes this the obvious target for diagnostic test development. Indeed, the abnormal 'scrapie' isoform of PrP, PrP^{Sc}, is to date completely specific for prion infection or disease [30–32]. Detection of disease-associated PrP in CNS and lymphoreticular tissues correlates widely with both the presence of the disease and the presence of prion infectivity [30–33]. The detection of abnormal PrP has been used diagnostically for several decades; originally for histopathological confirmation of prion disease postmortem and for the last 13 years for antemortem diagnosis from biopsied tissue, typically the tonsil. Developed for antemortem diagnosis of vCJD at the National Prion Clinic (London, UK), this has subsequently been included in the WHO's international diagnostic criteria for vCJD [34]. However, this involves a surgical procedure under general anesthesia and is only applicable where vCJD is suspected. In order to identify patients early it is critical to have a rapid and noninvasive test that can be considered by clinicians earlier in a differential diagnosis where CJD is a possibility.

Although the quantities of PrP^{Sc} deposited in neural tissues is sufficient during the presymptomatic and symptomatic phases of illness for detection by a range of conventional immunoassays such as western blot and ELISA, levels in peripheral tissues can be significantly lower [30–32,35]. However, transmission studies have demonstrated that prions, although at much lower concentrations, are present in peripheral tissues, including the blood, throughout the long presymptomatic phase [36–38].

The need for an early diagnostic tool is clearly focused on the analysis of an easily accessible biofluid, and the ease of access to blood samples would allow a test to be performed as soon as prion disease is considered by a physician, and even presymptomatically in at-risk individuals or blood donors. The measurement of the infectious agent itself or a close surrogate implies high specificity, but also offers a potential large dynamic response to experimental therapeutics targeting PrP, as the levels of deposited abnormal PrP and infectivity increase by many orders of magnitude during the latent phase of disease [39]. Specificity is a key parameter in achieving early diagnosis as the potential problems associated with a false-positive result will deter the use of any assay without clear clinical indications. It is equally important for the assay analyte to be readily accessible, and blood is the analyte of choice. It is conceivable that a blood-based assay for prion disease with sufficiently high specificity could be used not only for screening purposes within the National Blood and Transfusion Service (UK), but also as a routine test following neurology or psychiatry referrals. For an assay to be used so widely would typically require a specificity of at least 99.85% [40,41], which for prion disease effectively limits the molecular marker for infection to abnormal PrP.

Blood-based detection of abnormal PrP

There are a number of challenges associated with developing novel assays for the presence of abnormal PrP in the blood. PrP^{Sc} and related disease-associated abnormal forms of PrP are all conformers of a host-encoded protein PrP^C with an identical amino acid sequence, which do not contain nucleic acid and do not elicit a host humoral immune response. Thus, conventional high-sensitivity diagnostic methods such as the detection of an agent-specific immune response and nucleic acid detection by PCR are not applicable. Furthermore, the ratio of background

PrP^C to PrP^{Sc} is higher in blood than in CNS and lymphoreticular tissues with the high lipid and protein content of blood also potentially contributing to background signals.

Rodent models of prion disease have suggested that infectivity in the blood is low, with buffy coat fractions containing between 2 and 10 lethal doses (LD₅₀)/ml during the asymptomatic phases of the disease, rising to 100 LD₅₀/ml during the clinical phase [42,43]. More recently, experiments transfusing blood from experimentally infected sheep to previously healthy recipient animals, in parallel with transgenic mice, have indicated that the infectious prion titer in blood is greater than 10^{3.6} LD₅₀/ml and that the titer is significantly underestimated by intra-cerebral inoculation in rodent models [38]. However, this remains more than 5 logs below the level seen in CNS material and in order to successfully detect infection in the presymptomatic phase, a blood test must be able to detect PrP in a range that is orders of magnitude below the sensitivity of conventionally employed immunoassays.

There are a number of potential strategies for increasing the limit of detection of disease-associated PrP from a sample with low concentrations such as blood. Among these are techniques for the selective enrichment of abnormal PrP such as sodium phosphotungstic acid precipitation [35], immunoprecipitation [44] or capture by materials with high avidity for abnormal PrP such as stainless steel [18,45].

Alternatively, the self-replicating nature of prions can be exploited in various *in vitro* reactions that are capable of amplifying ‘seeds’ of abnormal PrP from low-to-detectable levels. The highest fidelity of replication and the ability to propagate infectivity is limited to protein misfolding cyclic amplification (PMCA) [46]. PMCA is a cyclical process analogous to PCR where there are periods of seeded conversion of substrate PrP^C by small amounts of PrP^{Sc} interspersed with bursts of sonication, which disrupt newly formed PrP aggregates, allowing for the continued recruitment and conversion of PrP^C. The only limiting factor to the level of amplification is the exhaustion of the substrate PrP^C or other factors necessary for amplification. PMCA has been used to detect PrP^{Sc} in buffy coat fractions from rodent blood [47,48], blood leukocyte preparations taken from scrapie infected sheep [49] and most recently to successfully detect infection in the whole blood of

experimentally infected rodents [50]. Although the extremely high sensitivity of PMCA suggests it is an attractive option as a diagnostic assay, there are some concerns over false-positive reactions [49,51], which coupled to its complexity and cost, make it more applicable as a confirmatory assay rather than a primary screening test.

A related group of amplification reactions based upon nucleated polymerization of recombinant PrP have been described where prion-infected tissues can seed the conversion of recombinant material into an amyloid form that is detected by the incorporation of an amyloid-specific fluorescent dye [52–54]. One derivative of this methodology, termed RT-QuIC, has been developed for use with CSF as an analyte for the diagnosis of sporadic CJD [55]. The assay performance characteristics are significantly better than current CSF markers for CJD infection such as 14-3-3 protein with a quoted sensitivity of 95% and a specificity of 99%. Although larger cohorts of samples need to be analyzed to confirm these performance parameters, this assay promises to be of great benefit in cases of rapidly progressive dementia where CJD is being considered and could replace the analysis of CSF 14-3-3, which is of limited value in more ambiguous clinical presentations.

Matrix capture & enrichment of abnormal PrP

To ensure the highest possible specificity for the detection of prion infection, carrying out an immunoassay for the presence of abnormal PrP offers the greatest potential [56]. Several studies contributed to a consensus that the majority of disease-associated PrP, although aggregated, is not resistant to methods traditionally used to degrade background, normal PrP^C as part of the postmortem diagnosis [57–64]. In the case of vCJD, our own work has shown that more than 80% of disease-associated PrP is sensitive to proteolysis [65]. Therefore, any blood-based assay for CJD infection must be able to differentiate abnormal PrP against a large background excess of PrP^C without using traditional methods of proteolytic digestion. One alternative approach is to specifically adsorb abnormal PrP from a complex mixture, an approach that also offers the potential for enrichment.

It has been known for several decades that prion infectivity can bind avidly to surgical grades of stainless steel and that the interaction is specific [18,45]. These findings have allowed the use of

appropriate steel matrices in the development of assays for prion infectivity with exquisite sensitivity that are in the correct range to detect blood-borne material [66]. Although highly specific and sensitive, this approach uses cultured cells and requires a period of weeks to complete. However, by adapting the matrix capture technology and coupling this to a conventional immunoassay with monoclonal antibodies directed against PrP, a rapid assay for disease-associated PrP has been developed that is capable of detecting vCJD infection in whole blood samples [67]. The specificity of this approach is extremely high, nominally 100% as previously reported. However, to confirm these initial estimates of specificity, a large cohort of control blood samples is required, which has been obtained from the USA where exposure to BSE is minimal and hence reactive samples should be absent. This specificity study is still in progress; however, several thousand controls have now been analyzed without any false-positive reactions.

This CJD blood test has also been in use at the National Prion Clinic since 2011 for validation purposes. During this time over 80 patient samples have been tested, the results from which have assisted with neurological diagnosis. In that time no positive test results have been found in nonprion disease patients and the test is now being applied to increasing numbers of cases.

Multiple & confirmatory assays

As blood testing for prion disease becomes a reality and increasing numbers of samples are analyzed, it is important to have additional assays available for the confirmation of test results. As previously discussed, RT-QuIC is a promising assay and could be used for the confirmation of initially positive results. The ‘gold standard’ confirmation for prion infection has always been regarded as animal bioassay where an experimental animal is inoculated with suspected material and succumbs to disease if the test sample is positive. Unfortunately, such experiments attract very significant costs and are time consuming, in some instances taking years to complete and as such are restricted to a research setting.

Due to the many drawbacks of bioassay research, effort has been invested in the development of cell culture-based assays for the quantitative measure of prion infectivity. Of the multiple cell lines isolated, only a minority are highly susceptible to prion infection and, thus, applicable to the estimation of prion titer. One

of the most widely used to study prion propagation is a mouse neuroblastoma-derived N2a cell line [68,69], but with a native susceptibility still significantly below that of whole-animal bioassay. However, successive rounds of selection and cloning from an N2a parent line have yielded a sensitive sub-clone designated PK1 [70] capable of detecting a 10^{-8} dilution of rodent brain, comparable to traditional bioassay. The isolation of this line has led to the development of robust assays for the rapid and accurate titration of prion infectivity [70] in various rodent tissues and has yielded the first highly sensitive assay for surface bound prions, the Standard Steel Binding Assay [66]. This assay has provided a means for the detection of surface bound prion infectivity to a 10^{10} -fold dilution of infected rodent brain; a detection limit greater than that of a conventional bioassay.

Such cell-culture models offer an alternative to whole animal bioassay while providing unambiguous results. Although at present these assays are restricted to the titration of rodent prions, research efforts are well underway to produce equivalent 'gold standard' confirmatory assays for human prions. The combination of rapid and specific primary blood tests for prion disease coupled to unambiguous confirmatory tests should allow the diagnosis of patients much earlier by removing the uncertainties of whether or not a test should be considered early in the differential diagnosis.

Conclusion & future perspective

The incidence of sporadic CJD is consistently one or two cases per million individuals per

annum and the current incidence of primary and secondary vCJD remains thankfully low despite an estimated infection prevalence in the UK of approximately one in 2000. However, the known, very long incubation periods of acquired human prion diseases [71] and the existence of powerful genetic susceptibility factors suggest that secondary infection may be occurring in recipients of transfused blood with clinical manifestation being an uncertain and long-term reality.

The demonstration that blood-based diagnosis is possible, at least in a subset of prion diseases, is likely to drive further developments in this area. This, coupled with numerous new technologies and techniques capable of providing confirmatory assays, makes it likely that routine testing for prion disease will become available. Ultimately, the availability of rapid and specific blood assays will allow the routine screening of blood donations as well as patients with even mild neurological or psychiatric impairment as soon as prion disease is suspected.

Financial & competing interests disclosure

J Collinge is a director and shareholder, and G Jackson is a shareholder of D-Gen Limited (London, UK), an academic spin-out company working in the field of prion disease diagnosis, decontamination and therapeutics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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