Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay

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Summary

Background Variant Creutzfeldt-Jakob disease (vCJD) is a fatal neurodegenerative disorder originating from exposure to bovine-spongiform-encephalopathy-like prions. Prion infections are associated with long and clinically silent incubations. The number of asymptomatic individuals with vCJD prion infection is unknown, posing risk to others via blood transfusion, blood products, organ or tissue grafts, and contaminated medical instruments. We aimed to establish the sensitivity and specificity of a blood-based assay for detection of vCJD prion infection.

Methods We developed a solid-state binding matrix to capture and concentrate disease-associated prion proteins and coupled this method to direct immunodetection of surface-bound material. Quantitative assay sensitivity was assessed with a serial dilution series of 10^{-7} to 10^{-10} of vCJD prion-infected brain homogenate into whole human blood, with a baseline control of normal human brain homogenate in whole blood (10^{-6}). To establish the sensitivity and specificity of the assay for detection of endogenous vCJD, we analysed a masked panel of 190 whole blood samples from 21 patients with vCJD, 27 with sporadic CJD, 42 with other neurological diseases, and 100 normal controls. Samples were masked and numbered by individuals independent of the assay and analysis. Each sample was tested twice in independent assay runs; only samples that were reactive in both runs were scored as positive overall.

Findings We were able to distinguish a 10^{-10} dilution of exogenous vCJD prion-infected brain from a 10^{-6} dilution of normal brain (mean chemiluminescent signal, $1{\cdot}3{\times}10^{5}$ [SD $1{\cdot}1{\times}10^{4}$] for vCJD *vs* $9{\cdot}9{\times}10^{4}$ [$4{\cdot}5{\times}10^{3}$] for normal brain; $p<0{\cdot}0001$)—an assay sensitivity that was orders of magnitude higher than any previously reported. 15 samples in the masked panel were scored as positive. All 15 samples were from patients with vCJD, showing an assay sensitivity for vCJD of 71·4% (95% CI 47·8–88·7) and a specificity of 100% (95% CIs between 97·8% and 100%).

Interpretation These initial studies provide a prototype blood test for diagnosis of vCJD in symptomatic individuals, which could allow development of large-scale screening tests for asymptomatic vCJD prion infection.

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Introduction

Prion disease encompasses various closely related and uniformly fatal neurodegenerative disorders affecting the CNS in human beings and animals. These disorders include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia and kuru in man, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease of deer and elk, and scrapie in sheep.[1,2] The emergence of variant CJD (vCJD) and the confirmation that it originates from exposure to BSE[3] has raised a plethora of public health concerns affecting endoscopy, surgery, dentistry, organ transplantation, and blood transfusion.

Exposure of the UK population to BSE has been widespread, with more than 181000 cases confirmed in cattle and estimates of total infections of 1–3 million.[4] Although the number of clinical cases of vCJD has been fairly small, the number of infected individuals remains unclear.[5] A retrospective study[6] of archived surgical lymphoreticular specimens estimated a prevalence of infection in the UK population of 237 per million (95% CI 49–692 per million), which is far higher than the number of clinical cases of vCJD thus far. On the basis of this study, a prevalence estimate of one in 4000 was recommended by the Spongiform Encephalopathy Advisory Committee for management of public health risks.[7,8] Further investigations have not refined these estimates,[9,10] and a major caveat to these studies is the unknown sensitivity of the methods used to detect subclinical or preclinical cases of vCJD infection. The long incubation periods seen in natural human prion infections, which can exceed 50 years,[11] and the existence of subclinical carrier states of prion infection in animal models[12] offer a potential explanation for the discrepancy between estimates of infection prevalence and clinical cases.

Concern regarding secondary transmission of vCJD has had extensive effects on public health policy in the UK and elsewhere. Since the prevalence of preclinical or subclinical vCJD prion infection is poorly defined, the extent of future transfusion-transmission of vCJD cannot be accurately risk-assessed. Secondary infection from asymptomatic donors has already been confirmed in four recipients of blood transfusion.[13–15] Although the number of transfusion recipients positively identified as having received packed red cells contaminated with vCJD is

small, a much larger cohort of around 7000 recipients of contaminated plasma products have already been identified and notified of their at-risk status.[16] Concern for this cohort has been heightened by post-mortem evidence of infection with vCJD prions in the spleen of an individual from this at-risk group with haemophilia.[17]

The infectious agents or prions responsible for transmission of disease are composed principally, if not entirely, of a misfolded form of host cellular prion protein (PrP^C). This unique pathogenesis has posed major challenges to the development of diagnostic tests, which for infectious agents generally involves detection of a host humoral immune response or agent-specific nucleic acid, neither of which apply in vCJD. PrP^C is expressed ubiquitously, although at highest concentrations in the CNS and cells of the immune system. When recruited during prion propagation, PrP^C is remodelled to aggregated, detergent insoluble isoforms designated PrP^{Sc}, which are chemically identical but conformationally distinct from PrP^C. Detection of PrP^{Sc} by the depletion of PrP^C with proteinase K (PK) in CNS and lymphoreticular tissues generally correlates with the presence of prion infectivity[18,19] and remains wholly specific for prion disease. However, in recent years, researchers have found that additional PK-sensitive but disease-related abnormal isoforms of PrP might have an important role in prion disease pathogenesis,[20,21] and only 10% of abnormal PrP may be deposited in the form of PrP^{Sc}.[22]

Although the quantities of PrP^{Sc} deposited in neural tissues are sufficient during the symptomatic phase of illness for detection by conventional immunoassays such as western blotting and ELISA, concentrations in peripheral tissues are substantially lower.[23] Quantification of infectious titre in rodent models has suggested that the levels of infectivity in blood are very low, with buffy coat fractions containing between 2 and 10 intracerebral LD_{50} units per mL during the asymptomatic phases of disease, rising to 100 LD_{50} units per mL during the clinical stage.[24,25]

To successfully identify infection in blood, an assay has to be able to detect abnormal PrP in a range that is several orders of magnitude lower than the sensitivity of conventionally used immunoassays. Furthermore, the ratio of background PrP^C (which is chemically identical to PrP^{Sc}) is higher in blood than in any other tissue, with the high lipid and protein content of blood potentially contributing to non-specific background signals.[26] Conventionally, immunoassays for PrP^{Sc} have depended on protease pretreatment of tissues to degrade PrP^C and other proteins, thereby reducing cross-reactivity.

The finding that prions can bind avidly to some surfaces, including metals,[27] has been used to develop quantitative assays for infectivity[28] that approach the high sensitivity needed to detect the low concentrations of prions and abnormal PrP associated with blood. We have adopted a similar approach for the capture and enrichment of abnormal PrP from whole blood using an optimised solid-state capture matrix derived from investigation of an extensive range of potential binding surfaces coupled with direct immunodetection of the surface-bound material. This approach avoids use of any proteolytic processing, ensuring that all abnormal PrP isoforms are available for detection. We aimed to establish the sensitivity and specificity of this test for detection of vCJD prion infection in blood.

Methods

Setting

These studies were approved by the local research ethics committee of the UCL Institute of Neurology and the National Hospital for Neurology and Neurosurgery (NHNN; London, UK). Blood samples were obtained with informed consent from patients who were enrolled in the MRC PRION-1 trial[29] or the National Prion Disease Monitoring Cohort study,[30] or who were referred to the National Prion Clinic or Dementia Research Centre at the NHNN. Samples were collected in EDTA blood tubes and stored frozen at –70°C. Diagnosis of variant or sporadic CJD was made according to established criteria.[31,32] Normal control blood samples were obtained as EDTA samples from the National Blood Transfusion Service (NBTS) of England and Wales.

Laboratory procedures

We assayed blood spiked with vCJD, blood spiked with normal brain homogenate, and whole human blood samples from patients. In the assay of spiked samples, an exogenous spike of 5 µL of 10% (w/v) vCJD-infected brain homogenate or normal human brain homogenate was diluted into 9995 µL of whole human blood. From this mixture, a serial dilution series of 10^{-7} to 10^{-10} of vCJD-infected brain homogenate into whole human blood was prepared, with a baseline control of a high concentration of normal human brain in whole blood (10^{-6}). Each sample was further diluted two-fold into capture buffer (200 mmol/L Tris, 4% [w/v] bovine serum albumin, 4% [w/v] CHAPS, 2×concentration of complete protease inhibitors [Roche, Mannheim, Germany], 80 units benzonase [grade II, Merck, Damstadt, Germany]) for assay. To aliquots of capture matrix (45 µm stainless steel particles [Goodfellow, Cambridge, UK]), 800 µL of the spiked blood-capture buffer mix was added and incubated overnight. In the assay of blood samples from patients, a whole human blood-capture buffer mix was added to aliquots of capture matrix and incubated overnight at 18°C with agitation.

After incubation with either spiked or whole blood, capture matrix was isolated on a magnet and the supernatant discarded. Capture matrix was washed repeatedly with 1 mL phosphate-buffered saline (PBS) plus 0·05% (v/v) Tween-20 (PBST). After the final wash, all liquid was removed and the capture matrix was heat-treated at 110°C for 5 min. To each tube, an aliquot of 50 µL of biotinylated primary antibody ICSM18 (D-Gen, London,

UK) prepared at 1 μg/mL in PBS plus 1% v/v Tween-20 was added and incubated at 37°C for 1 h. Samples were washed repeatedly with 1 mL PBST, isolating capture matrix each time. Each sample was then incubated with high sensitivity NeutrAvidin-HRP (Pierce, Rockford, USA) prepared at a 1:100 000 dilution in PBS plus 1% (v/v) Tween-20 at 37°C for 45 min.

Finally, samples were washed repeatedly with 1 mL PBST, isolating capture matrix each time. To each sample, 60 μL of SuperSignal ELISA Femto chemiluminescent substrate (Pierce) was added and 20 μL of capture matrix was transferred into three replica wells of a black flat bottom ELISA plate (Greiner, Frickenhausen, Germany). Immediately before plates were read, a further 80 μL of SuperSignal ELISA Femto chemiluminescent substrate was added per well. A dilution series of 1:100 000 to 1:10 million of the high sensitivity NeutrAvidin-HRP (Pierce) was prepared to allow for correction in absolute chemiluminescent readings across multiple plates. Plates were scanned with an M1000 plate reader (Tecan, Mannedorf, Switzerland).

Analyses

We analysed a panel of 190 whole blood samples, consisting of samples from 21 patients with vCJD, 100 normal controls (provided by NBST), 16 patients with probable sporadic CJD, 11 patients with definite sporadic CJD, and 42 patients from other neurodegenerative diseases (25 Alzheimer's disease, four frontotemporal dementia, six familial Alzheimer's disease, and seven neurological referrals to the National Prion Clinic subsequently confirmed as not prion disease). Samples were masked and numbered from 1 to 190 by individuals independent of sample collection, testing, or analysis. Each of the masked panel samples were tested twice in independent assay runs as described previously. Samples were processed and analysed in groups of 19 masked panel samples per 96-well plate with a set of eight quality control samples containing six normal control blood samples and two blood samples from patients infected with vCJD.

Masked panel samples were scored as reactive if the ratio of the mean chemiluminescence signal from three replicate wells exceeded a cutoff threshold determined for each plate. The threshold was set at the mean plus three SDs from the mean of the six normal blood samples on each plate. Thus, samples with a ratio of greater than 1 were regarded as reactive. To reduce potential false positive reactions, each sample was tested in two independent assay runs. Only samples that were reactive in both assays were scored as positive. On completion of testing of all samples in duplicate, the results were declared to an independent party and the samples decoded.

Instat (GraphPad Software, La Jolla, USA) was used for all analyses. Unpaired *t* tests, two-tailed, with Welch correction, were used for all comparisons.

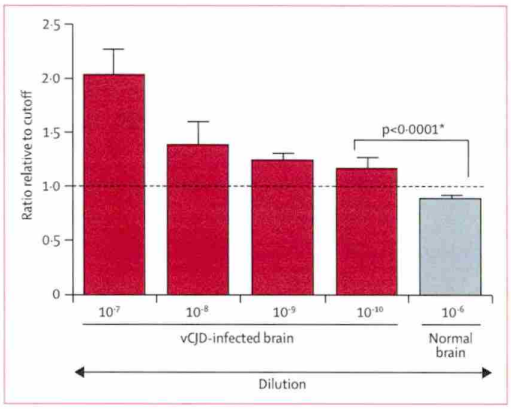

Figure 1: **Assay sensitivity to exogenous vCJD**
A serial dilution of vCJD prion-infected brain homogenate in whole human blood compared with a baseline control of normal human brain in whole blood; data shown are the mean of six replicates and are expressed as a ratio relative to the cutoff value (three SDs greater than the mean chemiluminescent signal for a normal brain sample; dashed line). Error bars show SDs. vCJD=variant Creutzfeldt-Jakob disease. *Two tailed, unpaired *t* test with Welch correction.

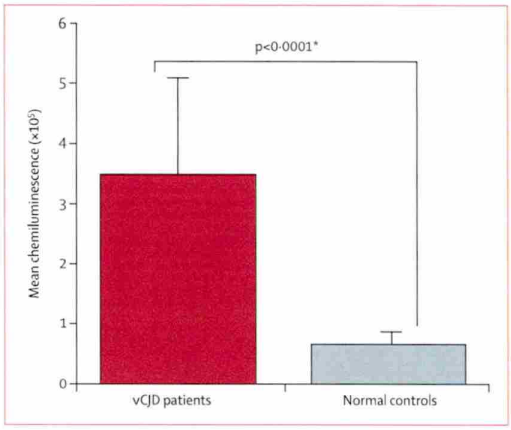

Figure 2: **Discrimination of endogenous vCJD blood samples from normal control samples**
Data shown are the mean chemiluminescent signal (arbitrary units) of 14 blood samples from individual patients with confirmed vCJD compared with the mean signal from 100 normal controls. Error bars show SDs for the mean signals. vCJD=variant Creutzfeldt-Jakob disease. *Two tailed, unpaired *t* test with Welch correction.

Role of the funding source

The sponsors provided financial support for the research but were not involved in the project design, interpretation of data or drafting of this manuscript. GSJ had full access to all the data in this study and had final responsibility for the decision to submit for publication.

Results

To establish the sensitivity of the assay compared with other methods, we analysed serial dilutions of vCJD brain homogenate diluted into whole blood. Exogenous

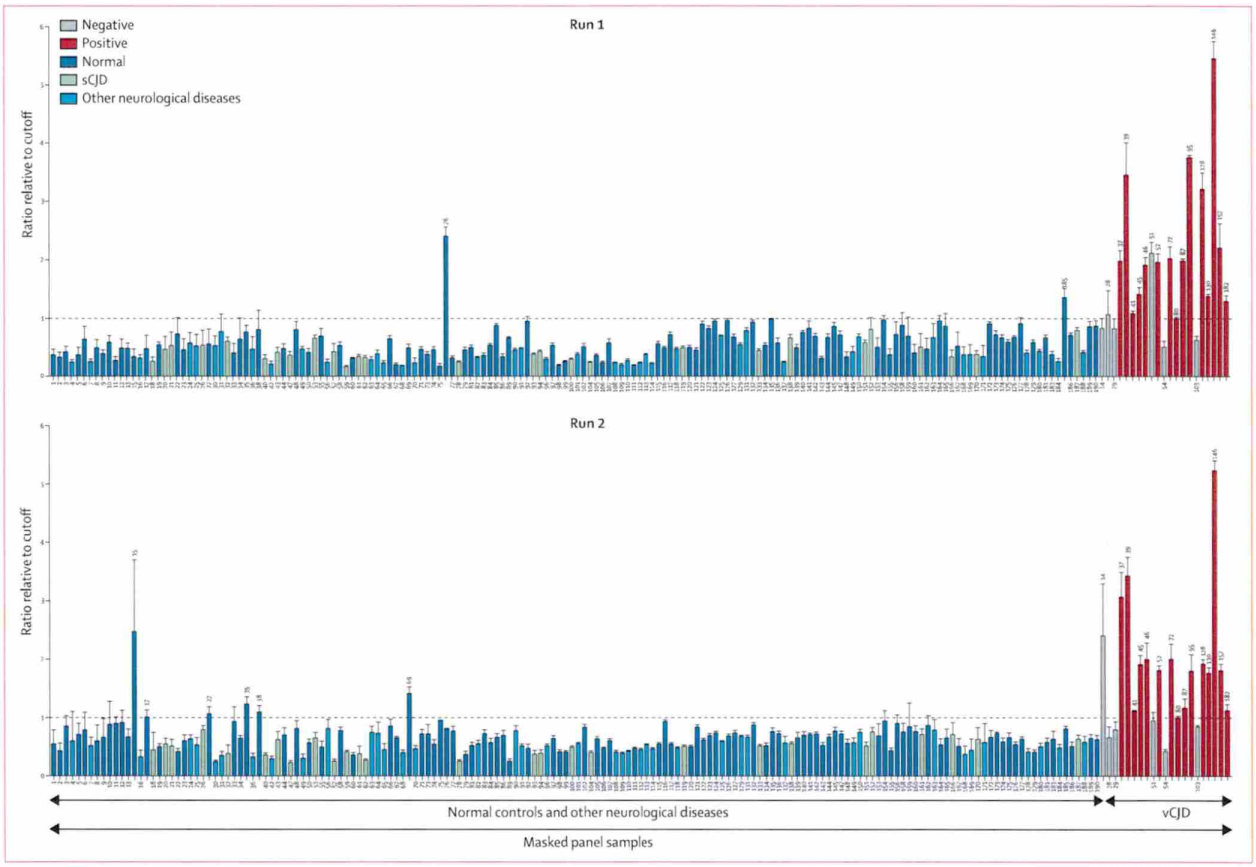

Figure 3: **Two independent assay runs for a masked panel of 190 blood samples**
The masked panel consisted of blood samples from 21 patients infected with vCJD, 100 healthy normal controls (dark blue bars), 27 patients with sCJD (green bars), and 42 controls with other neurological diseases (turquoise bars). Data are shown as the chemiluminescent signal ratio relative to a cutoff established as the mean of the signal for normal controls plus three SDs. Samples shown in grey are from patients infected with vCJD that had a ratio of less than 1 in one or both assays and were therefore scored as negative. Red bars show all samples that had a ratio greater than 1 relative to cutoff in both assays and were scored as positive. vCJD=variant Creutzfeldt-Jakob disease. sCJD=sporadic Creutzfeldt-Jakob disease.

spikes of vCJD-infected brain homogenate could clearly be distinguished from normal brain homogenate, even at a 10^{10}-fold dilution (figure 1), a sensitivity more than four orders of magnitude higher than previously achieved for immunoassay of vCJD tissue.[26] A chemiluminescent signal of mean $1{\cdot}3{\times}10^5$ (SD $1{\cdot}1{\times}10^4$) was obtained with 10^{-10} dilution of vCJD-infected brain versus $9{\cdot}9{\times}10^4$ (SD $4{\cdot}5{\times}10^3$) for a 10^{-6} dilution of normal control brain, corresponding to a ratio relative to the cutoff threshold of 1·2 (SD 0·1). The difference was highly significant ($p<0{\cdot}0001$). The highest dilution of vCJD brain could still be distinguished from the normal control when a threshold of mean normal signals plus three SDs was applied.

Initial studies of exogenous spikes of vCJD-infected brain homogenate in whole human blood showed that our assay was capable of discriminating between infected and non-infected samples with a sensitivity theoretically sufficient to detect infection in blood samples from patients with vCJD, on the basis of estimates of titre obtained from rodent models.[24,25] However, the biochemical nature of infectivity and abnormal PrP associated with blood is unknown. To ensure that this level of discrimination could be achieved with endogenous blood samples, we tested a subset of blood samples from 14 patients with confirmed vCJD from whom larger volumes of blood were available, and compared these with normal control blood samples (figure 2). The samples were analysed as groups and had mean chemiluminescent signals that were significantly different (normal, mean 65693 [SD 20538] *vs* vCJD, mean 348842 [160721]; $p<0{\cdot}0001$).

We then tested the masked panel of 190 samples from 21 patients with confirmed vCJD (including the 14 tested above), 69 patients with other neurological disease, and 100 normal healthy controls. 19 samples from the panel

were reactive in assay run 1, and 22 in run 2 (figure 3). A subset of 15 samples gave signals greater than the cutoff threshold in both assays and were hence scored as positive. Subsequently, all 15 samples were decoded as true positives obtained from patients with vCJD, showing an assay sensitivity of 15/21 or 71·4% (exact 95% CI 47·8–88·7).

Samples from six patients with vCJD did not score positive during testing of the masked panel. Of those samples, three had been reactive in either assay run 1 or 2 (figure 3), but did not react in both assays. The three remaining samples were scored as negative in both of the test runs and were indistinguishable from normal controls. Of the controls, eight samples gave single reactions (two in assay run 1, six in run 2). These eight reactions all came from normal controls obtained from the NBTS, with no reactions seen from sporadic CJD samples or samples from other neurological diseases. No samples other than those from patients with vCJD gave repeat reactive results and thus all of the normal and other neurological disease controls were correctly identified as true negatives (169/169), suggesting 100% specificity after two independent assays (exact 95% CIs between 97·8% and 100%).

Discussion

Evidence shows that there is a risk of iatrogenic (secondary) vCJD prion infection from transfusion of blood and blood products[13–15,33] and, by inference, from many forms of surgical and dental interventions. Taken together with the existence of subclinical carrier states of prion disease[12] and the long incubation periods for clinical disease,[11] the implications for current and future public health could be substantial. Current risk reduction strategies in the UK, of uncertain efficacy or necessity, are very costly to the National Health Service. These strategies include leucodepletion of all transfused packed red cells, sourcing of plasma from the USA, and importing of blood for transfusion to young children. Studies modelling the use of leucocyte depletion have shown reductions in blood infectivity of between 58% and 72%.[34,35] The use of prion blood filters is also being considered by the UK Department of Health.[36]

Our research efforts have focused on sensitive methods for detection of vCJD prion infection in whole blood, since this sample is not only easily obtained for clinical purposes, but is also the target for protection of the donated blood supply. Application of a capture matrix for enrichment of abnormal PrP and optimised immunodetection of bound material has allowed detection of vCJD brain homogenate diluted 10^{10}-fold in exogenous spiking experiments (figure 1). This concentration of abnormal PrP is about 100 000 times lower than is encountered during tonsil biopsy and high sensitivity western blotting[23] currently used at the National Prion Clinic for diagnosis of vCJD. It is also 10 000-fold beyond the most sensitive reported immunoassays for vCJD,[26] and is more sensitive than conventional rodent bioassays that typically do not report dilutions of prion-infected brain tissue greater than 10^8-fold for rodent-adapted scrapie strains, and which are far less sensitive for assay of human vCJD prions because of transmission barrier effects.[37] Although this finding might at first seem contradictory, rodent bioassays report infectious titre and do not detect PrP molecules directly. The number of PrP monomers that constitute an infectious particle is not defined and indeed is likely to be heterogeneous.[38] However, a single infectious unit contains many PrP monomers, as an aggregate all of which are potentially available to our assay, allowing sensitivity beyond that previously achieved.

As a rare disease, blood samples from patients with vCJD are inevitably scarce, and validation of assay results with the high numbers of samples conventionally expected is impossible. We have applied our assay to samples of whole blood that were readily available and for which sufficient volumes were present to prevent significant depletion of this scarce resource. 21 samples were tested, of which 15 were positively identified in our masked panel, yielding a sensitivity of 71%. Although this finding is a very significant step forward in prion diagnostics, it should be interpreted within the context of associated specificity. Indeed, for consideration as a screening assay of blood and tissue donations, specificity is expected to exceed a minimum of 99·85% to avoid large numbers of false positive tests.[39]

Our panel contained 100 normal blood samples from the NBTS and 69 neurological disease controls. We included samples obtained from patients with Alzheimer's disease because, in addition to being an important differential diagnosis in suspected CJD, abnormal PrP deposition can accompany amyloid β accumulation in these diseases.[40] In addition to Alzheimer's disease, other disorders that form part of the differential diagnosis for prion disease were also included in the panel, including frontotemporal dementia, young onset familial Alzheimer's disease, and cases referred to the National Prion Clinic as suspect cases, but for whom prion disease was later excluded as a diagnosis. Encouragingly, none of this small series of neurological disease controls provided any reactions in either of the two independent assay runs. The single reactive samples were all normal controls obtained from the NBTS. The most likely explanations for occasional reactions in the assay are cross-contamination or incomplete removal of primary antibody during washing. For these reasons, samples were assayed twice and had to be reactive in both assays to be deemed positive. Although no false-positive results were recorded by our assay criteria (0/169), the exact 95% CIs for specificity are 97·8% to 100%. This level of specificity would be acceptable in neurological diagnostic use, but screening of large (>10 000) numbers of negative controls from countries with low BSE prion exposure is necessary to

accurately determine specificity before considering the assay's wider application as a screen of asymptomatic individuals.

Our testing has to date been restricted to samples from patients with clinical vCJD. Serial blood samples are being obtained under the auspices of the National Prion Disease Monitoring Cohort[10] from several vCJD prion-exposed at-risk individuals who might become symptomatic in the future and on whom longitudinal clinical and neuroradiological studies are being performed. Such samples could in due course provide data for the stage at which prionaemia is detectable by our or other future tests. Notably, evidence from rodent models of prion infection suggest involvement of blood at early preclinical stages.[41] Extended longitudinal study of individuals testing positive will be needed to establish the proportion of patients with vCJD prionaemia that go on to develop clinical vCJD and how many are chronic carriers.[42]

Despite these uncertainties, our findings demonstrate the ability to detect prion infection in blood and show that a donor blood screening test is technically feasible. The use of this test in differential diagnosis of suspected vCJD will be further investigated in large case series.

Contributors

JAE and GSJ designed the experiments with contributions and direction from JC. MF, AS, and PT assisted in development of the methods and analysed all samples with JAE. JB, TC, JL, SM, PR, and JC investigated the patients and obtained, processed, and documented clinical samples. JAE, JC, and GSJ drafted the report.

Conflicts of interest

JC is a director and JC and GSJ are shareholders and consultants for D-Gen (London, UK), an academic spinout company working in the field of prion disease diagnosis, decontamination, and therapeutics. D-Gen markets the antibody used in this study. All other authors declare that they have no conflicts of interest.

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