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RESEARCH ARTICLE

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Enhanced Creutzfeldt-Jakob disease surveillance in the older population: Assessment of a protocol for screening brain tissue donations for prion disease

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Abstract

Human prion diseases, including Creutzfeldt-Jakob disease (CJD), occur in sporadic, genetic, and acquired forms. Variant Creutzfeldt-Jakob disease (vCJD) first reported in 1996 in the United Kingdom (UK), resulted from contamination of food with bovine spongiform encephalopathy. There is a concern that UK national surveillance mechanisms might miss some CJD cases (including vCJD), particularly in the older population where other neurodegenerative disorders are more prevalent. We developed a highly sensitive protocol for analysing autopsy brain tissue for the misfolded prion protein (PrP^{Sc}) associated with prion disease, which could be used to screen for prion disease in the elderly. Brain tissue samples from 331 donors to the Edinburgh Brain and Tissue Bank (EBTB), from 2005 to 2022, were analysed, using immunohistochemical analysis on fixed tissue, and five biochemical tests on frozen specimens from six brain regions, based on different principles for detecting PrP^{Sc}. An algorithm was established for classifying the biochemical results. To test the effectiveness of the protocol, several neuropathologically confirmed prion disease controls, including vCJD, were included and blinded in the study cohort. On unblinding, all the positive control cases had been correctly identified. No other cases tested positive; our analysis uncovered no overlooked prion disease cases. Our algorithm for classifying cases was effective for handling anomalous biochemical results. An overall analysis suggested that a reduced biochemical protocol employing only three of the five tests on only two brain tissue regions gave sufficient sensitivity and specificity. We conclude that this protocol may be useful as a UK-wide screening programme for human prion disease in selected brains from autopsies in the elderly. Further improvements to the protocol were suggested by enhancements of the in vitro conversion assays made during the course of this study.

KEYWORDS
brain bank; neurodegenerative diseases, Creutzfeldt-Jakob disease; prion; surveillance; variant CJD (vCJD)

Richard Knight and Marcelo A. Barria share senior authorship.

1 | INTRODUCTION

Human prion diseases are rare, invariably fatal neurodegenerative diseases associated with the conversion of the normal prion protein (PrP^{C}) to an abnormal, misfolded, form (PrP^{Sc}), which accumulates mainly in the central nervous system (CNS). Human prion diseases have sporadic, acquired, and genetic aetiologies; Sporadic Creutzfeldt-Jakob disease (sCJD) is the commonest form, with a worldwide annual mortality rate of 1–2 per million. A polymorphism encoding either methionine (M) or valine (V) at codon 129 of the prion protein gene *PRNP* affects susceptibility to disease and phenotypic variability. Molecular subtypes of human prion diseases are based on the combination of codon 129 and the western blot profile of protease-resistant PrP^{Sc} ($resPrP^{Sc}$) and are associated with different age and clinicopathological profiles. The median age of death for sCJD is 68 years, but human prion diseases have a wide age range, and individuals at the extreme ends of this range potentially pose more of a diagnostic challenge [1].

Variant Creutzfeldt-Jakob disease (vCJD) is an acquired form of human prion disease that originated from bovine spongiform encephalopathy (BSE) dietary contamination and was notable for affecting younger individuals (median age at death 28 years). vCJD is characterised by extensive deposition of PrP^{Sc} in non-CNS lymphoid tissues. As of March 2023, there have been 178 definite and probable cases of vCJD in the United Kingdom; 175 of primary, dietary, cause and three resulting from red blood cell transfusions [2–5]. There are two instances of transmitted infection (the infected recipients dying without developing vCJD); one from red cell transfusion and one considered to be due to Factor VIII treatment [6, 7]. Given the long incubation period of BSE/vCJD, and the possibility that some of those infected may never develop clinical disease, there are likely to be individuals with asymptomatic vCJD in the UK population.

Estimates of the prevalence of asymptomatic vCJD infection in the UK population have been made using the analysis of routine surgical lymphoid (mostly appendix) specimens for PrP^{Sc}, suggesting a figure of around 1:2000 [8–10]. With a significant BSE dietary exposure of the UK population and the suggested prevalence of asymptomatic infection, further vCJD cases have been expected dietary, with long incubation periods, and secondary, via blood from asymptomatic donors [11]. However, no vCJD cases have occurred in the UK since 2016, and no blood-transmissions of vCJD since 2008 [12], despite the well-established UK CJD surveillance system and its associated projects (such as the UK Transfusion Medicine Epidemiology Review) [3, 13, 14]. There are several possible explanations for this, including case under-ascertainment, particularly in the elderly. It is notable, and unexplained, that the annual mortality rate of sCJD generally rises with age but then falls off in the very elderly; it potentially reflects case under-ascertainment in this age group. A similar case under-ascertainment may occur for elderly patients with genetic forms of prion disease normally associated with a younger profile compared to sCJD. A progressive neuro-cognitive illness is relatively unusual in the young and very likely to lead to neurological referral and detailed investigation. However, in the elderly, neurodegenerative diseases (such as Alzheimer's disease) are common, and detailed neurological investigation may not occur. In addition, sCJD affects the elderly; vCJD might present differently in older people and might be diagnosed as the commoner sporadic form although the available evidence does not suggest that vCJD is clinically different in the elderly [15–17]. There are methodological problems in trying to find possibly missed cases in the elderly, with a detailed clinical and autopsy investigation of all elderly cognitive diseases presenting great difficulties. A national study of progressive intellectual and neurological deterioration (the PIND study), looking for potential missed vCJD cases in children, was possible given the relative rarity of this clinical presentation in the very young [17–19]. In parallel to our laboratory study reported, a clinical feasibility study of 'atypical' cognitive illness in the elderly in one region (Lothian)-which, here, will be referred to as the '65+ Study'-met with limited success [20].

Our study aimed to establish a protocol for in-depth screening of banked brain tissue for unrecognised prion disease (either symptomatic or asymptomatic) in people aged 65 years and above. The current internationally agreed diagnostic criteria for prion disease involves clinical, neuropathological, immunohistochemical, and biochemical assessments [21, 22]. A tissue protocol for detecting clinically unsuspected (including asymptomatic) prion disease, including potentially novel prionopathies, needs to involve very sensitive PrP^{Sc} or $senPrP^{Sc}$ (protease-sensitive forms) detection methods, in addition to the routine histological, immunohistochemical, and Western Blotting techniques. Four additional, published, biochemical test protocols, for the direct and indirect detection of the disease-associated prion protein, were considered: sodium phosphotungstic acid precipitation/western blotting (NaPTA), conformation-dependent immunoassay (CDI), real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA). NaPTA is based on the concentration and detection of protease-resistant PrP^{Sc} ($resPrP^{Sc}$); it proved effective in detecting low levels of $resPrP^{Sc}$ in the spleens of asymptomatic patients exposed to vCJD infection, through blood transfusion or blood products [6, 23, 24]. CDI detects PrP^{Sc} based on the detection of concealed epitopes that are exposed when PrP^{Sc} is denatured; it is not dependent on the protease resistance of PrP^{Sc} and, therefore, can detect $senPrP^{Sc}$ [25, 26]. Neither NaPTA nor CDI have been assessed in routine diagnostic use or brain tissue screening.

The other two methods are based on the amplification of misfolded PrP and have the potential to detect extremely low levels of PrP^{Sc}. RT-QuIC uses incubation and shaking to mimic and accelerate prion replication in vitro using recombinant PrP substrate and can detect extremely low levels of sCJD PrP^{Sc} in cerebrospinal fluid (CSF) and nasal lavages [27–30]. However, RT-QuIC is less well able to detect the PrP^{Sc} found in vCJD [29]. PMCA uses incubation and sonication to effect prion replication in vitro using brain PrP^{C} substrate. PMCA is ultra-sensitive for detecting low levels of vCJD PrP^{Sc} in patient blood [31, 32], urine [33] and CSF [34], but it is less efficient at amplifying sCJD PrP^{Sc} [34, 35]. Applying these two complementary conversion methods offers the prospect of screening for very low levels of abnormal prion protein in patients who are symptomatic or asymptomatic, regardless of aetiology [36].

This study evaluated all five biochemical tests in parallel as tools for screening brain tissue from a cohort of neurological patients, aged 65 or more, who had donated their tissue to the Edinburgh Brain and Tissue Bank (EBTB); their comparative effectiveness in this role has not been previously addressed.

2 | MATERIALS AND METHODS

2.1 | Inclusion criteria

All donations (fixed or frozen brain tissue) to the EBTB, made between April 2005 and July 2022, from individuals aged 65 or more at death, were eligible for this study, and none of these patients had been considered for a diagnosis of prion disease. EBTB is part of the UK Brain Bank Network, providing high-quality post-mortem materials for diagnosis and research into disorders of the brain and nervous system, and receives donations from several national and local research studies in Scotland [37, 38]. We received donations from Alzheimer Scotland, the Scottish Motor Neurone Disease (MND) Register and the Lothian study of IntraCerebral Haemorrhage Pathology, Imaging and Neurological outcome (LINCHPIN) [39–41] and the Lothian '65+ Study' [20] studies. Collectively, these form a selected patient group with non-CJD neurodegenerative conditions, amongst which missed prionopathy might be found. All donations had appropriate consent and ethical approval for retention and research, and accompanying basic clinical data.

2.2 | Study design

The overall design of this study has been described previously [42]. Two samples were defined: the 'retrospective' and the 'prospective'.

The 'retrospective' samples consisted of donations received by EBTB between April 2005 and March 2015, along with a blinded panel of confirmed prion disease cases (from the NCJDRSU CJD Brain and Tissue Bank). The blinded positive control cases included two vCJD cases (MM and MV at codon 129), two cases of growth hormone-associated iatrogenic CJD (iCJD; MV2 and VV2), a range of sCJD subtypes (MM1, MV1, VV1, MM2, MV2, VV2), a case of variably protease-sensitive prionopathy (VPSPr), and cases of genetic forms of human prion diseases: Gerstmann-Sträussler-Scheinker disease associated with P102L, and familial CJD associated with E200K. These cases were used to validate our test protocols and to assess an algorithm for classifying the biochemical panel results. An algorithm was used to ensure the specificity for detecting human prion disease; this being necessary because the high analytical sensitivity of these techniques might produce occasional false positive results. Samples might test negative, positive, or anomalous (e.g., an unusual or novel pattern of WB bands). If a sample initially tested anomalous or positive, repeat tests were performed to establish a final classification of the case according to this decision algorithm as described previously [42], as 'negative', 'negative-anomalous' or 'positive' for prion disease.

The established protocol, with the algorithm, was then applied to the 'prospective' samples, which consisted of tissues received between April 2015 and July 2022. All biochemical analyses were performed in a Category 3 (with derogation) containment laboratory with strict adherence to health and safety protocols. DNA was extracted from grey matter-enriched frozen brain tissue samples (20–30 mg) of the frontal cortex for *PRNP* codon-129 genotype analysis, performed by restriction fragment length polymorphism analysis as described previously [43].

2.3 | Biochemical analysis

Where available, frozen brain tissue from six regions (frontal, parietal, temporal, and occipital cerebral cortex, cerebellum, and thalamus) were used, and in nearly all cases at least frontal cerebral cortex and cerebellum were analysed.

2.3.1 | Standard Western blotting (WB)

Samples of frozen brain tissue (100 mg) were homogenised and digested with proteinase K using the standard method used at NCJDRSU for post-mortem diagnosis of patients with suspected prion disease [44], but including the modifications described by Parchi et al. to aid molecular subtyping [45, 46]. In addition, 50 μL samples of the PK digested extracts were centrifugally enriched to maximise detection sensitivity for $resPrP^{Sc}$ [47]. For each western blot, a vCJD brain positive control standard (5 μL of a 10% brain homogenate, equivalent to 500 μg of tissue, PK-digested, not centrifugally concentrated) and a non-CJD negative control (50 μL of a 10% brain homogenate,

equivalent to 5 mg of tissue, PK-digested, centrifugally concentrated) were analysed in parallel with the test samples. Immunodetection was performed using the monoclonal antibody 3F4 (Millipore, Watford, UK, MAB1562) as described previously [46].

A positive result was defined as the appearance of three bands in the 18–30 kDa range or at least two bands co-migrating with bands in the vCJD positive control or alternatively, a single band co-migrating ~8 kDa, in the presence or absence of slower migrating bands.

2.3.2 | Sodium phosphotungstic acid (NaPTA) precipitation/Western blotting (WB)

For high-sensitivity detection, enrichment of PrP^{Sc} by sodium phosphotungstic acid (NaPTA) precipitation, prior to WB analysis, was carried out as previously described [7, 24, 26]. Western blotting was performed as described above, except that the detection reagent used was SuperSignal West Femto maximum sensitivity substrate (Thermofisher). A positive result was defined as described for standard WB (see above).

2.3.3 | Conformation-dependent immunoassay (CDI)

The CDI assay was performed and calibrated as described previously [25, 26] (but omitting the NaPTA precipitation step). PrP was detected using the dissociation-enhanced lanthanide fluorescence immunoassay technology of PerkinElmer (PerkinElmer, Cambridge, UK). The capture antibody used was MAR-1 (0.5 μg/well), provided by CSL Behring, Marburg, Germany. The detection antibody was 0.2 μg/mL biotin-conjugated 3F4 (BioLegend), used in combination with 0.2 μg/mL Europium-labelled streptavidin (PerkinElmer). In CDI, $senPrP^{Sc}$ is detected based on an increase in signal following denaturation of the sample (D) with guanidine hydrochloride (GdnHCl), compared with non-denature (N) samples, However, brain homogenates were assayed by CDI following limited proteolytic digestion with a low concentration of PK (2.5 μg/mL, 1 h, 37°C) to minimise the background signal, without digesting $senPrP^{Sc}$ [26, 48].

Test samples were continually analysed in comparison to three non-CJD neurological control cases and one vCJD case. A cut-off threshold was established based on the mean [D–N] value for non-CJD neurological control test samples plus three standard deviations of the mean.

2.3.4 | Real-time quaking-induced conversion (RT-QuIC)

RT-QuIC was performed as described previously, with minor modifications [29, 30]. The 100 μL RT-QuIC reactions were set up in duplicate in the wells of a clear-bottom black 96-well microplate (Fisher Scientific, UK). The RT-QuIC reactions were initiated by the addition of 2 μL of a 10^3-fold dilution of the 10% (w/v) homogenate. This amount is equivalent to 2×10^{-7} g brain (wet mass) per 100 μL reaction. The tissue samples were tested in parallel with two positive control standards (sCJD MM1 and sCJD VV2) and non-CJD neurological control samples from three patients considered for a diagnosis of CJD but given an alternative diagnosis. A positive conversion reaction was defined as a Thioflavin T fluorescence reading three times greater than the average reading at 0.5 h (the baseline) for both duplicate reactions, for three successive 15 min readings within 100 h after the start of the reaction.

2.3.5 | Protein misfolding cyclic amplification (PMCA)

PMCA was carried out as described previously [35, 46]. However, in this study the test sample brain homogenates themselves were used as a source of PrP^{C} for the conversion to PrP^{Sc}, thus avoiding the necessity of matching the *PRNP*-codon 129 genotype of the conversion substrate with the seed. Test samples were subjected to PMCA, alongside positive control reactions in which vCJD brain was diluted 1:100 in human brain homogenate from a non-CJD neurological control patient (MM at *PRNP*-codon 129). Low molecular weight heparin (100 μg/mL) was included as a cofactor in all PMCA reactions. A total of 96 PMCA cycles were performed comprising 20 s of sonication followed by 29 min, and 40 s of incubation per cycle. The samples were then analysed by Western blotting, and the results were classified, as described above.

2.4 | Assigning molecular subtypes

In the event of detecting a case that was positive for prion disease by WB or NaPTA/WB, an attempt was made to assign a molecular subtype defined on *PRNP* codon 129 genotypes, and the $resPrP^{Sc}$ WB profile. The latter was assigned according to the nomenclature of Parchi et al. [49].

2.5 | Neuropathological analysis

All histological and immunohistochemical analysis on formalin-fixed paraffin embedded tissue was performed as described previously [46]. Frontal cortex and cerebellum were immunolabelled with two monoclonal anti-PrP antibodies recognising different epitopes of the prion protein: 12F10 (amino acids 142–160, Bioquote Ltd, UK) and KG9 (amino acids 140–180, TSE Resource Centre, Roslin Institute, UK).

TABLE 1 Basic data for all the cases ($n = 331$), and cases with available frozen tissue analysed using the biochemical tests in this study ($n = 274$) not including positive controls.

		Total number of cases, (all with fixed tissue available)		Subset with frozen tissue for biochemical analysis	
		Total ($n = 331$)	Percentage	Total ($n = 274$)	Percentage
Sex	Male	180	54.1	124	45.2
	Female	151	45.6	150	54.7
Age at death (years)	65–69	55	16.6	49	17.9
	70–74	69	20.8	60	21.9
	75–79	68	20.5	54	19.7
	80–84	65	19.6	51	18.6
	85–89	46	13.9	36	13.1
	90+	28	8.5	24	8.7
Source	65+ study	12	3.6	12	4.4
	Alzheimer's Scotland	41	12.4	40	14.6
	Procurator Fiscal Cases[a]	59	17.8	49	17.9
	Hospital consented research PM	9	2.7	6	2.2
	LINCHPIN	129	39.0	88	32.1
	Lothian birth cohort	18	5.4	18	6.6
	MND Register	51	15.4	49	17.9
	MS tissue bank	2	0.6	2	0.7
	Misc. Neuropath condition	6	1.8	6	2.2
	PSP Study	4	1.2	4	1.5
PRNP codon 129	MM			108	39.4
	MV			131	47.8
	VV			34	12.4
	Not determinable			1	0.4

[a]Cases where a post-mortem had been ordered by the Scottish Procurator Fiscal to investigate a sudden or unexplained death.

3 | RESULTS

A total of 331 patients were included in the study who had donated tissue to EBTB, with 173 used in the retrospective arm of the study and 158 used in the prospective arm of the study. A summary of these patients' details, showing the frozen and fixed tissues that were available, and the source studies, is provided in Table 1. There was a slight preponderance of males in the study cohort, which was seen for all source studies of donated tissue used in the current study, except the LINCHPIN study, which provided slightly more female patients than males. The distribution of *PRNP* codon 129 genotypes in the study cohort aligned with the codon 129 distribution previously reported for the UK population [50].

For the majority of cases (274) frozen tissue was available. Cases that had fixed tissue, but lacked frozen tissue for biochemical analysis, tended to be from the LINCHPIN study, or cases where a post-mortem had been conducted to investigate a sudden or unexplained death (Procurator Fiscal cases). It was not possible to perform biochemical or genotype analysis for the cases that lacked frozen tissue. The age profile and sex ratio of the whole cohort did not differ from the subset of patients within this cohort with available frozen tissue.

TABLE 2 Retrospective cases ($n = 173$) and blinded positive controls ($n = 13$), showing the numbers of fixed and frozen tissue samples that were available.

Fixed tissue regions	Frozen tissue regions available (usually frontal cortex and cerebellum	Numbers of cases: Retrospective cohort ($n = 173$) and blinded controls ($n = 13$)
2	2[a]	101
	1	24
	0	56
1	2	1
	1	3
0	2	1
	Total	186

[a]For one of these cases, frozen parietal cortex was provided for analysis instead of frozen frontal cortex.

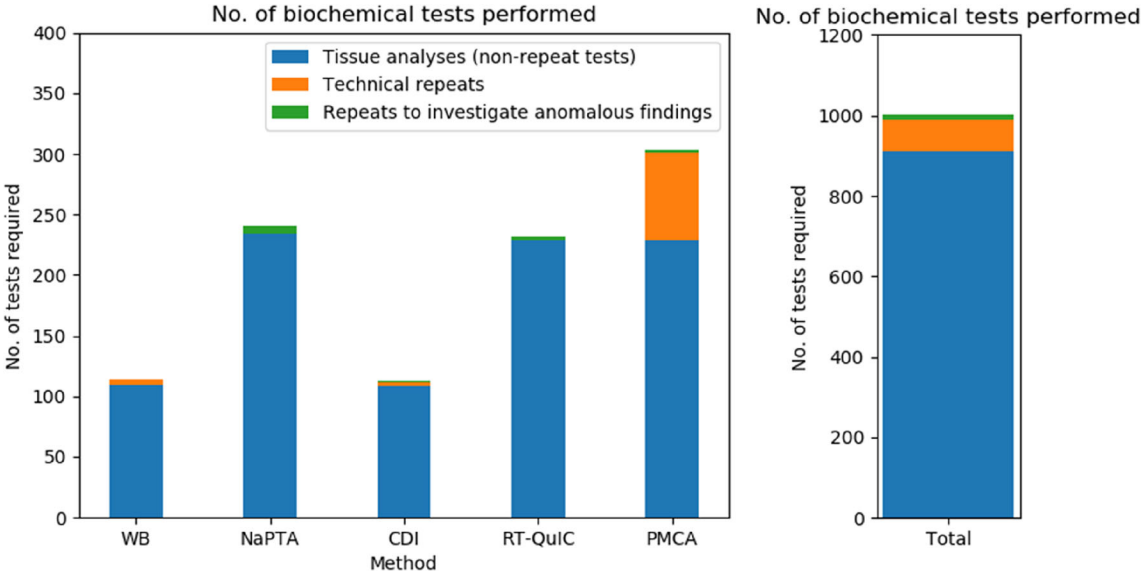

FIGURE 1 Number of biochemical tests performed for the analysis of 130 retrospective cases with frozen tissue, including the blinded controls. The numbers shown include the numbers of repeats performed due to technical failures (orange) and to investigate anomalous findings (green).

3.1 | Retrospective cases and blinded controls: Summary of results

Just over half (173) of the 331 recruited patients were analysed in the retrospective arm of this study. Of these 117 had frozen tissue available for biochemical analysis, in addition to fixed tissue available for histopathological/immunohistochemical analysis (Table 2). In addition to these cases, planted within the retrospective cohort of assumed negative case tissues, was a panel of 13 blinded prion disease positive control cases.

All 13 of these were correctly identified using the biochemical test protocol, including definite cases of sporadic CJD and VPSPr, vCJD and iCJD, and genetic forms (in five cases, there was insufficient tissue to complete all five biochemical tests). The results are summarised in Table S1. Furthermore, all 12 positive controls with available fixed tissue were identified by histopathological/immunohistochemical analysis following microscopic examination. (One of the 13 blinded test panel cases lacked fixed tissue.)

Frozen brain tissue was available for biochemical analysis from 117 of the 173 cases in the retrospective cohort, with none of the 117 cases being classed as positive for any of the biochemical tests. Consistent with the biochemical observations, the 172 cases of the retrospective cohort with available fixed tissue showed no neuropathological features of prion disease.

3.2 | Performance of the algorithm for biochemical analysis

A total of 1002 biochemical tests were performed in the retrospective arm of the study, including technical repeats (Figure 1). Using the algorithm described by us previously, of cases in the retrospective arm of the study with frozen tissue available, 13 cases were classed as positive (blinded controls), 112 as negative, and five as 'negative-anomalous'. These latter five cases were classed as such because they had initially tested positive by either RT-QuIC (3 cases), CDI (1 case), or NaPTA (1 case), but repeat tests were negative.

3.3 | Biochemical test protocol sensitivity

All of the positive controls, and none of the retrospective cohort cases, identified as prion disease. However, not all the biochemical tests were positive for all of the tissues (Table S1). The sensitivities of the neuropathological analysis and the individual biochemical tests were calculated, and are shown in Table S2. The results showed that of the biochemical tests, only NaPTA was 100% sensitive, and standard western blotting least sensitive, at 86.4%. The two in vitro conversion systems, RT-QuIC and PMCA, showed a sensitivity of 87% and 95.5%, respectively. WB was unable to identify a case of sCJD (case 12) with a relatively uncommon molecular subtype (VV1). NaPTA and CDI analyses of this case were positive but confirmed that the levels of PrP^{Sc} associated with this case were very low. CDI was unable to detect PrP^{Sc} in the frontal cortex tissue from a case of VPSPr (case 2), possibly because the low molecular mass PrP fragment associated with the cerebral cortex of VPSPr cases lacks the epitope recognised by the MAR-1 antibody used in CDI. In contrast, WB and PMCA were both able to detect PrP^{Sc} from the frontal cortex, but not the cerebellum from this case. PMCA was also unable to amplify

PrP^{Sc} from the parietal cortex of one case of sCJD with an MV2 molecular subtype. However, it was able to amplify the two vCJD cases, even when the cases were either MM or MV at *PRNP* codon 129.

RT-QuIC was positive in all CJD cases, including the fCJD and GSS cases, except- as expected—in vCJD, where it was less sensitive. For one of the two vCJD control cases, a positive RT-QuIC result was obtained from cerebellum, but not frontal cortex tissue; for the other case neither frontal cortex, nor cerebellum tissue, was positive.

From standard WB and NaPTA precipitation, it was possible to determine the $resPrP^{Sc}$ molecular subtypes of the positive control cases, as either 1 or 2 (molecular mass 21 kDa and 19 kDa, respectively). There was generally good agreement between our, and the previously established classifications of the cases (detailed in Table S3). It was also possible to correctly determine the expected glycosylation ratios of the $resPrP^{Sc}$ products for 8 of 13 cases. In one case, our study found type 1, whereas the established classification was type 2 sCJD. However, this may be explained by neuropathological screening indicating a mixed $resPrP^{Sc}$ type.

TABLE 3 Cases analysed in the prospective study ($n = 158$) and frozen tissue samples that were available.

Tissue regions	No. cases
6	106
5[a]	2
2	49
0	1
Total	158

[a]For two cases no frozen thalamus samples were available.

3.4 | Refinement of biochemical test protocol

WB and CDI were discontinued because neither technique had detected PrP^{Sc} in tissues that had not already been identified as positive by NaPTA. RT-QuIC and PMCA were continued potentially to detect low levels of PrP^{Sc} in sporadic and genetic CJD (RT-QuIC) or vCJD (PMCA).

3.5 | Prospective cases

A total of 158 cases were included in the prospective arm of the study. Frozen tissue was available for all but one these cases for the biochemical protocol, and fixed tissue from frontal cerebral cortex and cerebellum was available for all 158 cases for PrP immunohistochemistry (Table 3). A total of 4682 biochemical tests were done (including repeats; Figure 2). Overall, there were repeat tests that resulted either due to technical failures (mostly, of the internal positive control for amplification in PMCA in the original test), or the investigation of an anomalous finding.

None of these 158 cases was classified as prion disease by the neuropathological assessment of fixed tissue. For frozen tissue, none of the prospective cases were classified finally as 'positive' for any of the biochemical tests, but 23 cases were assigned as 'negative-anomalous finding' according to the algorithm for classifying cases. Most of

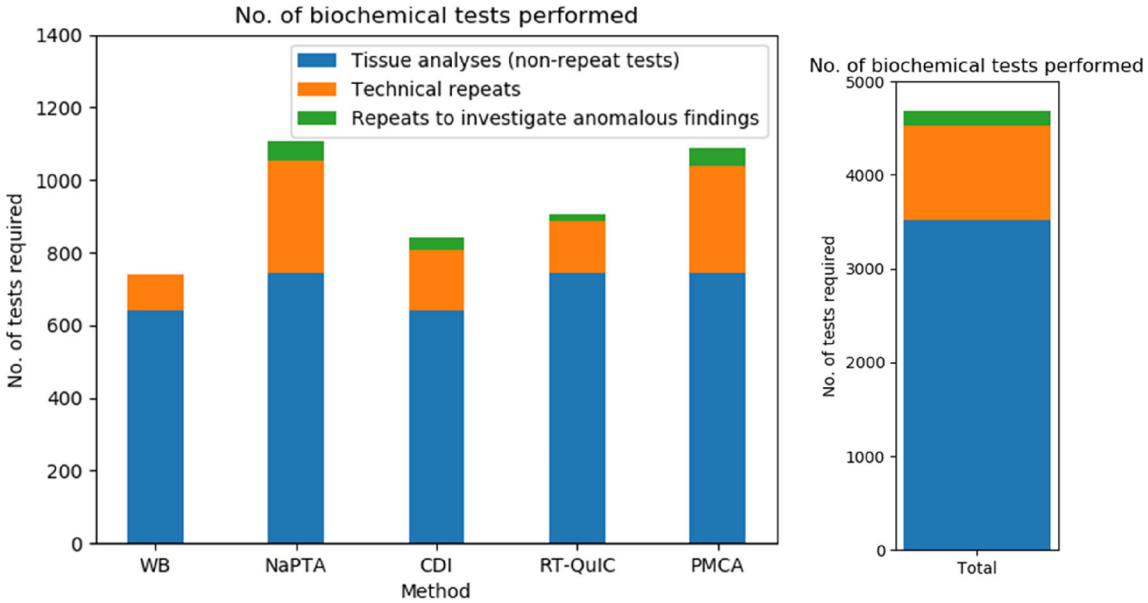

FIGURE 2 Number of biochemical tests performed for the analysis of 157 prospective cases with available frozen tissue. The numbers shown include the numbers of repeats performed due to technical failures (orange), and to investigate anomalous findings (green).

these were due to samples that initially tested positive on RT-QuIC, but on further analysis were negative. Some NaPTA tests were either initially positive but negative on repeat testing or produced a faint banding pattern that was not consistent with known forms of $resPrP^{Sc}$. One case gave positive results for both NaPTA and RT-QuIC, but repeat tests were negative, and no other tests indicated evidence of prion disease for this case. In one case, anomalous findings were observed for more than one biochemical test (see Table S4). No clear link was established between classification as 'negative–anomalous finding' and study source, sex, *PRNP* codon 129, post-mortem interval, or weight or pH of the brain taken at autopsy.

3.6 | Assessment of throughput

Time taken to conduct neuropathological and biochemical (NaPTA, RT-QuIC, and PMCA) analyses on the two brain regions: frontal cortex and cerebellum tissue, using a pre-set number of cases was determined. The date the tissue samples were transferred to the NCJDRSU laboratory as the start-point, with the date of reporting the full set of analyses as the endpoint. In terms of staffing, two staff members conducted the biochemical analyses, and one performed the neuropathological analysis. During a period of 74 days, 94 tissues, from 48 cases (retrospective), were fully analysed. The total number of biochemical tests performed in this period, including repeats, is shown in Figure S1. During this assessment, technical difficulties were experienced with the PMCA test, due to a failure of the amplification controls. From our assessment of throughput for our screening protocol, we conclude that it would be possible to fully analyse ~20 cases (~40 tissue specimens) per month, using the three essential biochemical tests, in combination with neuropathological analysis based on three full-time staff.

4 | DISCUSSION

The aim of this study was to evaluate a protocol for screening brain tissue donations from individuals who are aged 65 or above, with a neurological or neuropathological diagnosis, for evidence of undetected prion disease (symptomatic or asymptomatic). In the course of this evaluation, we have not uncovered any missed cases of human prion disease, including vCJD, in a cohort of more than 300 patients, nor any evidence of asymptomatic vCJD infection. However, we have developed and tested a protocol of histopathological and biochemical tests, which could be suitable for the analysis of larger cohorts of patients from the United Kingdom as a whole.

The study involved analysis of tissues from a 'retrospective' group (including donated tissues, positive and negative controls), followed by a refinement of the protocol and then an application to a 'prospective' group of donated brain tissue. The first phase involved five biochemical tests (four of which had not been used in routine brain tissue prion diagnosis); these were reduced to three after the initial evaluation (NaPTA, RT-QuIC, and PMCA). For brain tissue screening, standard WB and CDI tests do not increase the overall sensitivity of a protocol that includes NaPTA/WB. Moreover, all the positive prion disease controls would have been detected from an analysis of tissue from frontal cortex and cerebellum alone, even with the omission of CDI and standard WB. These factors justify the reduction of the test panel to two of the initial six brain tissue regions. A further option could be to omit NaPTA because, in concert, PMCA and RT-QuIC detected all positive control cases. Rationalisation of the number of tests and tissues increases the potential of this protocol to be applied to a larger number of cases.

We employed an algorithm for dealing with any anomalous test results, allowing us to define criteria for a final positive result [42]. In terms of classification of cases by biochemical analysis, the algorithm proved to be highly effective. Of 117 retrospective cases with available frozen tissue that were not part of the blinded positive controls, 112 were classified as negative, and 5 as negative-anomalous. In the prospective arm of the study, of the 158 cases, 135 cases were negative and 23 were negative-anomalous. Apart from standard WB, all biochemical tests identified all 13 of the blinded positive control cases (a broad spectrum of prion disease subtypes). Standard WB failed to identify one of the sCJD cases (VV1) as a prion disease, but our NaPTA and CDI analysis, and a review of the original diagnostic western blots, showed that the $resPrP^{Sc}$ levels, in this case, were low. NaPTA/WB was the only biochemical test that showed 100% sensitivity and specificity, in terms of the results obtained for both cases and tissues. CDI, which exploits differences in the conformation of PrP^{Sc} versus PrP^{C} to detect protease-sensitive PrP^{Sc}, did not provide any clear advantages over NaPTA, or the other tests, in terms of sensitivity, and was unable to detect PrP^{Sc} in the frontal cortex of a case of VPSPr, which is associated with PrP^{Sc} with a lower protease resistance. As expected, RT-QuIC was effective at detecting all subtypes of prion disease, except for vCJD. In fact, it was surprising that RT-QuIC was able to detect one of the vCJD cases, possibly owing to the relatively high titre of PrP^{Sc} associated with this case. In contrast, PMCA could amplify PrP^{Sc} from all subtypes tested except sCJD MV2A. The utilisation of PMCA also permitted the identification of the two vCJD cases, even though one of these cases was the only known definite vCJD case with an MV codon 129 genotype. This is consistent with our previous observations that our PMCA method preferentially detects vCJD compared to sCJD, and can detect both MM and MV vCJD [34]. Although the blinded panel did not include cases of sCJD

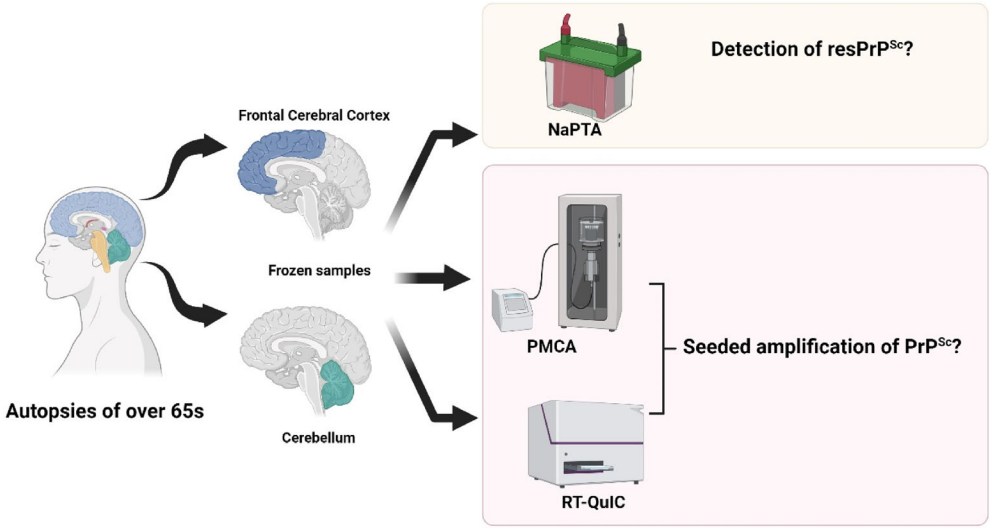

FIGURE 3 Flow diagram for proposed protocol for enhanced CJD surveillance.

MM2 Thalamic subtype, or fatal familial insomnia, previous work gives us confidence that the lower levels of resPrP^{Sc} in the frontal cerebral cortex from these patients would still be detectable by NaPTA/WB [51].

The primary aim of our protocol was to test for any evidence of prion disease in the cases analysed, and not the sub-classification of molecular subtypes. Nevertheless, there was agreement between our determination of molecular subtype for the blinded panel of prion disease and their known molecular subtype, with only a single exception which possibly reflected the co-occurrence of both subtypes in this case, a known phenomenon in sCJD [52, 53].

In both the blinded prion disease samples, and the other cases, the biochemical protocol test results were consistent with the neuropathological analysis. However, the blinded panel cannot not account for the possibility of novel, or asymptomatic cases with very low levels of PrP^{Sc} in the CNS. There are currently no such cases with available tissue for further analysis.

It should be noted that, during the conduction of this study, there have been significant advances in PMCA and RT-QuIC technology. Advances in PMCA have enabled detection of vCJD PrP^{Sc} in blood, including in preclinical disease [31, 32, 54] and CSF [34, 55]. Our own modified version, hsPMCA, can now detect minute amounts of PrP^{Sc} in the CSF of vCJD cases, including patients who are MM or MV at *PRNP* codon 129 [34]. A second-generation form of RT-QuIC has been developed that can detect PrP^{Sc} in sCJD patient CSF with equivalent sensitivity and specificity to the original version, but with a significantly shorter assay turn-over time [56–58]. Ideally, any future screening study should exploit these advancements.

The biochemical test protocol, and the algorithm for classifying cases, were effective in the analysis of 158 prospective cases, received after April 2015. None of these cases, including the five 65+ study participants, were positive. However, 23 cases were classified as negative-anomalous. Most anomalous results were either positive, but non-reproducible RT-QuIC results, with relatively long lag times, which might be able to be discounted by refining future criteria for RT-QuIC positivity. Other anomalous results were NaPTA analyses with banding patterns that were faint and non-reproducible or did not match the pattern expected for known prion disease subtypes. Atypical WB banding patterns are less easily discounted since they have in the past proved key in identifying novel prion diseases [59, 60].

An important consideration of a brain screening protocol is throughput i.e. the number of cases that can be analysed within a given amount of time. According to our assessment of throughput, using this protocol, we could perform this analysis on just over 20 cases per month with three dedicated staff members. This estimate may be somewhat conservative, owing to some technical difficulties that were experienced with PMCA in the course to the throughput analysis. However, while these difficulties were fully resolved, it is always possible that other technical problems might arise with these techniques affecting throughput.

Other studies on the ascertainment of prion disease have been carried out for other populations: In a US study, seven out of 6000 dementia patients in a national database were clinically unsuspected but autopsy-confirmed prion disease cases [61]. However, it is difficult to know whether this result could be extrapolated straightforwardly to the United Kingdom, with its different neurology service system, its well-developed national CJD surveillance programme, and the likely greater awareness of CJD in the wake of the BSE epidemic.

5 | CONCLUSION

Due to the long incubation periods of prion diseases, and the potential for cases to my masked by the high incidence of dementia in the older population, prion disease

surveillance remains a public health priority. This study shows that prion disease surveillance could be enhanced, based on the analysis of autopsy brain samples, using three biochemical tests (NaPTA, PMCA, and RT-QuIC), on two brain regions (frontal cortex and cerebellum; Figure 3). This protocol for enhanced prion disease surveillance also has relevance for countries other than the United Kingdom. Monitoring the emergence of novel genetic, iatrogenic, or environmentally acquired prionopathies is important for global public health [62]. However, while this protocol could aid prion disease detection, addressing the question of possible underascertainment of prion disease in the elderly would still require an ambitious approach (such as examining a substantial selection of all autopsies of the over 65 s). To be manageable, potentially a further adjustment of the protocol would be required, including the newer optimised version of PMCA and the second-generation RT-QuIC with a shorter analytical time.

AUTHOR CONTRIBUTIONS

This study was designed by Anna Molesworth, Alexander H. Peden, Richard Knight, and Marcelo A Barria. The experiments were designed and conducted by Adriana Libori, Alexander H. Peden, Helen Yull, and Diane L. Ritchie. Patient information was supplied by Colin Smith. The data were analysed, and the manuscript was written by Alexander H. Peden, Lovney Kanguru, Diane L. Ritchie, Richard Knight, and Marcelo A. Barria. All authors contributed to the editing and revision of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analysed during the current study are not publicly available due patient confidentiality but are available from the corresponding author on reasonable request.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments. EBTB has ethical approval to provide tissue samples to research projects (REC reference 16/ES/0084), including those for pilot studies. For this type of study formal consent is not required. This article does not contain any studies with animals performed by any of the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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