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Research Article

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Abstract

Background: Recently, the investigation of cerebrospinal fluid (CSF) biomarkers for diagnosing human prion diseases (HPD) has garnered significant attention. Reproducibility and accuracy are paramount in biomarker research, particularly in the measurement of total tau (t-tau) protein, which is a crucial diagnostic marker. Given the global impact of the coronavirus disease pandemic, the frequency of measuring this protein using one of the world's fully automated assays, chemiluminescent enzyme immunoassay (CLEA), has increased. At present, the diagnosis and monitoring of neurological diseases mainly rely on traditional methods, but their accuracy and responsiveness are limited. There is a limited knowledge on the accuracy of CLEA in Tau measurements. We aimed to measure t-tau protein using CLEA and to elucidate its merits and limitations.

Methods: We analysed CSF samples obtained from 91 patients with rapidly progressive dementia using ELISA and CLEA. Additionally, we used western blotting to detect the presence of 14-3-3 protein and employed real-time quaking-induced conversion (RT-QuIC) assays to analyse the same set of samples. Furthermore, we examined the correlation coefficient between ELISA and CLEA results in a subset of 30 samples. Moreover, using CLEA, we evaluated the diurnal reproducibility, storage stability, dilutability, and freeze-thaw effects in three selected samples.

Results: Among the 91 patients, a total of 45 (22 men and 23 women) tested positive for HPD in the RT-QuIC assay. In contrast, all CSF samples from the remaining 46 patients without HPD (23 men and 23 women) tested negative in the RT-QuIC assay. Both ELISA and CLEA showed perfect sensitivity and specificity (100%) in measuring t-tau protein levels. Furthermore, there was a strong correlation coefficient ($R^2 = 0.9363$) between ELISA and CLEA results. However, despite its advantages, CLEA analysis exhibited instability for certain samples with t-tau protein levels exceeding 2,000 pg/mL, leading to low reproducibility during dilution analysis.

Conclusions: Our findings indicate that CLEA outperforms ELISA in terms of diurnal reproducibility, storage stability, and freeze-thaw effects. However, ELISA demonstrated superior performance in the dilution assay. Therefore, it is imperative to develop innovative approaches for the dilution of biomarker samples for CLEA measurements during clinical trials.

Background

Human prion disease (HPD), a fatal and incurable neurodegenerative disorder, is attributed to the misfolding of prion protein (PrP). Prion diseases encompass a phenotypically diverse spectrum of conditions characterised by aberrantly conformed protease-resistant proteins. These disorders can be classified into three types: sporadic forms (including Creutzfeldt-Jakob disease (CJD), sporadic fatal insomnia, and variably protease-sensitive prionopathy); genetic forms (including genetic CJD, fatal familial insomnia, and Gerstmann-Straussler-Scheinker syndrome); and acquired forms (including kuru, variant CJD, and iatrogenic CJD) [1].
In the past, the diagnosis of HPD relied on clinical manifestations and electroencephalogram (EEG) criteria [2] because of the inability to detect abnormal prion protein without brain biopsy. Therefore, additional methods are required to diagnose HPDs. The clinical diagnosis is facilitated by the detection of biochemical markers in the patient’s cerebrospinal fluid (CSF). Western blotting is considered a reliable diagnostic tool for HPD because it enables the detection of total tau (T-tau) and 14-3-3 proteins in the CSF [3].

ELISA has been predominantly used to quantify Ab42, T-tau, and P-tau181 [4]. This technique involves manual or semi-automated addition and removal of reagents on a microplate using an ELISA processor. However, its widespread implementation in routine clinical laboratories has faced significant challenges [5]. It is important to highlight the lack of standardization, awareness regarding pre-analytical importance and specimen management, as well as analytical factors that may affect the final results. While this method has been used in non-neurological clinical settings with consistent detection measures in serum [7-9], there have been limited studies investigating its accuracy for biomarker detection in CSF and scarce research comparing methods and diagnostic performance on novel innovative platforms.

Despite being the most commonly employed technique for detecting core biomarkers in the CSF of patients with HPD, the ELISA method often results in substantial interlaboratory and intralaboratory variability, which limits their widespread adoption in clinical practice. To address this issue, a novel diagnostic approach was integrated with the ELISA detection system to improve the early diagnosis of HPD, which is crucial for effective disease prevention [10-11].

Chemiluminescent enzyme immunoassay holds immense potential to revolutionize clinical practice and research, serving as valuable indicators of normal or pathological biological processes and dynamic responses to therapeutic interventions. In a clinical setting, it can aid in diagnosis, disease activity monitoring, and evaluation of treatment impact on clinical response. At the scientific research level, it provides crucial selection information for observational and experimental studies while also serving as an alternative, secondary or even primary endpoint in trials. While neurospecific liquid chemiluminescent enzyme immunoassays are currently lacking, the diagnosis and monitoring of neurological disorders rely heavily on neurological examinations, clinical assessments, outcome scoring, and neurophysiology; furthermore, peripheral nerve imaging techniques are continuously improving despite their limited functionality. Traditional methods offer a high degree of semi-quantification but lack accuracy when identifying potential abnormalities; they fail to differentiate between residual damage and active disease while exhibiting poor responsiveness. Specific fluid-based chemiluminescent enzyme immunoassays can simplify tasks such as diagnosing, predicting, and monitoring under active states. Moreover, with an increasing number of reported HPD emerging rapidly, there is an urgent need for sensitive and reliable biomarkers that personalise treatment approaches while enhancing cost-effectiveness [12]. The immunochromatographic assays and chemiluminescent enzyme immunoassays (CLEAs), produce faster results than polymerase chain reaction (PCR) [13,14]. We frequently use CLEAs for antigen and antibody testing because of their demonstrated utility. CLEAs provide advantages, such as simultaneous reproducibility, intraday reproducibility, and excellent storage stability.
By contrast, the approval of drugs for Alzheimer’s disease in the U.S. and Japan has increased the significance of using spinal fluid biomarkers to diagnose this condition. CLEA, which is known for its superior reproducibility compared to that of ELISA, can be employed to quantify CSF biomarkers [15].

We aimed to use CLEA to measure t-tau protein levels in CSF samples and demonstrate its utility for assessing tau levels in patients with HPD. In addition, we aimed to elucidate the strengths and weaknesses of CLEA.

**Methods**

**Aim, design, and setting of the study**

In this 10-year prospective study (2011–2020), we aimed to demonstrate the utility of CLEA for assessing t-tau levels in the CSF samples of patients with HPD and elucidate its strengths and weaknesses. CSF biomarker analysis was conducted on 3,000 patients with rapidly progressive dementia at Nagasaki University. The Ethics Committee of the Nagasaki University Graduate School of Biomedical Sciences approved the study protocol (ID No. UMIN000038398, UMIN000016855, and UMIN000003301). All patients or family members of patients provided written informed consent.

**Patients**

Of the 3,000 patients with rapidly progressive dementia, 91 patients were randomly selected for further investigation. The diagnosis and subsequent analysis in all patients were validated by the Japanese Surveillance Commission. PrP genotyping was performed using genomic deoxyribonucleic acid (DNA) extracted from peripheral blood leukocytes.

**Biochemical analysis of CSF samples (t-tau protein assay kit (CLEA) Lumipulse®)**

Imprecision and linearity of CLEA by the Lumipulse® (Fujirebio Inc., Tokyo, Japan)

Imprecision was assessed by analysing low- and high-quality control samples 10 times within the same analytical run (repeatability) or 30 times over a period of 15 days (reproducibility). Mean, standard deviation (SD), and coefficient of variation (CV) were calculated. For linearity evaluation, a CSF sample with a high concentration of t-tau protein was serially diluted using the diluent provided by the manufacturer.

**CLEA measurement of t-tau protein in the CSF of 91 patients**

CLEA (t-tau protein assay kit Lumipulse®) was employed to quantify t-tau protein in the CSF according to the manufacturer's instructions. The procedural steps involved in this analysis were as follows. The total tau calibrator or specimen, along with a biotinylated antibody solution, was added to the particle solution. Subsequently, t-tau presents in the specimens or calibrators specifically bound to the anti-t-tau monoclonal antibody (mouse) on the particles, as well as to the biotinylated antibody (mouse). This
binding led to the formation of biotinylated antibody-antigen immunocomplexes. The particles were thoroughly washed and rinsed to eliminate any unbound material. Alkaline phosphatase (ALP)-labelled streptavidin was selectively attached to the biotinylated immunocomplexes on the particles. A final round of washing and rinsing was performed to remove any remaining unbound materials that were mixed with the substrate solution, wherein the ALP-conjugated particles catalysed the dephosphorylation of the adamantyl-1,2-dioxetane phosphate (AMPPD) present in the solution. This enzymatic reaction leads to the generation of luminescence at a maximum wavelength of 477 nm upon the cleavage of dephosphorylated AMPPD. The luminescent signal serves as an indicator for quantifying the t-tau protein levels across all samples.

**Ethical Statement**

The Ethics Committee of the Nagasaki University Graduate School of Biomedical Sciences approved the study protocol (ID No. UMIN000038398, UMIN000016855, and UMIN000003301). All patients or family members of patients provided written informed consent. All samples were analysed blinded for at least personal data.

**ELISA of t-tau protein in the CSF of 91 patients**

Cerebrospinal fluid samples from all 91 patients were subjected to T-tau detection following the manufacturer's instructions. The analysis was performed simultaneously on four different plates (all from the same batch, n, 23J12AA), with an equal distribution of CJD and non-CJD samples in each plate. Protein Western Blot signals were used to determine sample dilution empirically. Positive samples were diluted at a ratio of 1:20 using the provided dilution buffer, weak positive samples at a ratio of 1:5, and negative samples also at a ratio of 1:5. The diluted samples were then added to wells coated with specific antibodies against 14-3-3c and incubated for one hour at room temperature. After four wash steps, they were further incubated with anti-14-3-3c detection antibody (diluted at a ratio of 1:100) according to the manufacturer's instructions for another hour at room temperature. Following additional washing steps, HRP-conjugated anti-IgG secondary antibody (diluted at a ratio of 1:100) was added and the samples were incubated for another hour at room temperature. Unbound HRP conjugates were washed away before reacting with substrate hydrogen peroxide-tetramethylbenzidine solution. The reaction was stopped by adding an acidic solution, and absorbance of the resulting yellow product was measured using a microplate reader (Multiskan Ascent V 1.24) set to read absorbance values at 450 nm wavelength. A standard curve was generated by plotting absorbance against T-tau concentration units using calibrator values, enabling the determination of arbitrary concentration units (AU/mL) for unknown samples based on this standard curve range between 125-16000 AU/mL in human cerebrospinal fluid.

**Biochemical analysis of CSF samples (t-tau protein by ELISA and 14-3-3 protein using Western blotting and RT-QuIC assay)**

T-tau protein in the CSF was measured using ELISA following the manufacturer's instructions. An identical standard was used for all the experiments. Western blotting was performed to measure the 14-3-
3 protein levels, as previously described. The RT-QuIC assay was performed using recombinant human PrP by following established protocols. All CSF samples collected from patients suspected of having HPD were stored as aliquots at -80°C until use. Assays were performed once every 2 weeks to minimise repeated freeze-thaw cycles. Haemorrhagic CSF samples were excluded from the study.

Samples were simultaneously tested six times at three concentrations (low, medium, and high) using the lumipulse G600II system and the experimental reagent 6BX2101 for detecting T-tau protein levels. The obtained values for each sample were recorded to assess reproducibility. The coefficient of variation (CV) for the control group ranged from 1.8 to 3.7%, while the CV for the experimental group ranged from 1.2 to 2.3%. To evaluate day-to-day reproducibility, measurements were taken twice daily over five days by testing both control and experimental samples ten times at three concentrations (low, medium, high). In the control group, CV ranged from 2.6 to 3.2%, whereas for the experimental group it ranged from 1.9 to 4%.


The study design was to compare the reproducibility, stability and effectiveness of CLEA and RT-QuIC as diagnostic methods of human viral diseases.

To compare the specificity, sensitivity, and predictive values of CLEA and RT-QuIC methods, we analysed cerebrospinal fluid samples from 88 patients with Prion disease and 84 control patients.

To assess the repeatability of CLEA and RT-QuIC methods, we simultaneously tested samples from 91 sCJD patients in a subset of 30 samples to examine the correlation coefficient between ELISA and CLEA results.

To evaluate the stability of CLEA and RT-QuIC methods, sCJD cerebrospinal fluid samples (n=9) were incubated under short-term (0–7 days), medium-term (0–14 days), and long-term conditions (0–29 days) at extremely low temperature (-80°C), low temperature (-20°C), room temperature (4°C). We studied the detection values of tau protein using both detection methods [15].

Whole Blood Influence: To assess the impact of blood contamination on comparing CLEA and RT-QuIC in CSF samples, to investigate the influence of whole blood on T-tau measurements, two random samples were divided into three portions resulting in a total of six experimental samples with varying percentages of whole blood (0, 1, and 10%). After thorough shaking, T-tau values were measured using both CLEA and ELCIA methods revealing an increase in T-tau values ranging from 100 to 107% as blood volume increased.

We added varying proportions (0, 1, 10%) of blood cells into sCJD (n=3) and control (n=3) CSF samples. We studied the detection values of tau protein using both detection methods.
To investigate the effect of transportation storage on sample testing accuracy, freeze-thaw experiments were conducted on cerebrospinal fluid samples (n=3), with multiple cycles performed for dissolution.

Statistical analysis

Using IBM SPSS Statistics 25, statistical analysis was conducted on the sample test data. Using the statistical software, we carried out the normal distribution verification, pair comparison, multivariate test and other methods to calculate the test data. The results are represented by a regression line and equation, wherein the intercept quantifies constant error while the slope measures proportional error. The 95% confidence intervals (CIs) of both the intercept and slope in the regression equation are employed to evaluate potential systematic differences and proportional disparities between these two methods. Furthermore, the slope and intercept elucidate whether values exhibit solely random discrepancies, thereby enabling consistent conclusions to be drawn from both methodologies. The results were as follows:

- In terms of reproducibility, the standard deviation (SD) ranged from 10.7 to 26.4, with a coefficient of variation (CV) ranging from 1.2 to 3.7%, consistently remaining below the threshold of 4.2.

- For future reproducibility, the SD values ranged from 10.1 to 31.7, with a CV range between 1.9 and 4.2%, indicating consistent CV values below the threshold of 5.3.

- Regarding storage stability at different temperatures, t-tau protein measurements remained relatively stable for up to 4 days at -20°C and -80°C but decreased by approximately 5% after storing for 29 days compared to measurements taken at 14°C.

- Dilution capability varied among different types; however, most dilution ratios fell within the range of 43 to 49%, with deionized water being closest to a 50% dilution ratio.

- Resistance against blood contamination: Even in cases where there is up to 10% whole blood contamination present, CLEIA detection remains highly sensitive in identifying cerebrospinal fluid samples with variations lower than CV (3%).

Results

Patients profiles

Among the 91 patients, 45 (22 men and 23 women) were identified as having HPD by positive reactions in the RT-QuIC assay (See Additional Table 1a and b, Additional File 1). The remaining 46 patients (23 men and 23 women) were classified as non-HPD patients because of negative reactions in the RT-QuIC assay (Additional Table 1a and b, Additional File 1). Furthermore, we conducted a second-generation QuIC assay on all samples following the Additional Methods (Additional File X), which yielded results consistent with our RT-QuIC assay findings [16]. The patients were diagnosed and categorised according to the new diagnostic criteria for sporadic HPD [17-18]. The classification of these 45 patients with HPD
was based on both the WHO criteria and the aforementioned new criteria, and they were confirmed as having typical and classical sporadic CJD. All patient diagnoses and subsequent analyses were validated by the Japanese Primary Disease Surveillance Commission.

**Simultaneous reproducibility of t-tau protein measurements with CLEA in the same aliquots of the control and test samples**

The initial sample was partitioned into six distinct aliquots for simultaneous measurements across three control and test samples. The SD ranged from 10.7 to 26.4, signifying that fluctuations exceeding a magnitude of 1,000 pg/mL in t-tau protein measurements by CLEA within this range of SD values were observed among the samples. Furthermore, the CV ranged from 1.2 to 3.7%, consistently maintaining levels below the threshold of 4%.(Table 1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control samples</th>
<th>Test samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>1</td>
<td>311</td>
<td>511</td>
</tr>
<tr>
<td>2</td>
<td>320</td>
<td>535</td>
</tr>
<tr>
<td>3</td>
<td>330</td>
<td>533</td>
</tr>
<tr>
<td>4</td>
<td>342</td>
<td>546</td>
</tr>
<tr>
<td>5</td>
<td>325</td>
<td>539</td>
</tr>
<tr>
<td>6</td>
<td>346</td>
<td>532</td>
</tr>
<tr>
<td>Number</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Min</td>
<td>311</td>
<td>511</td>
</tr>
<tr>
<td>Max</td>
<td>346</td>
<td>546</td>
</tr>
<tr>
<td>Mean</td>
<td>329.0</td>
<td>532.7</td>
</tr>
<tr>
<td>SD</td>
<td>12.1</td>
<td>10.7</td>
</tr>
<tr>
<td>CV (%)</td>
<td>3.7</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 1. Simultaneous reproducibility in the same aliquots of the control and test samples

Control samples: The content of T-tau protein in all samples was detected by ELISA.

Test samples: The content of T-tau protein in all samples was detected by CLEA.

Abbreviations: SD, standard deviation; CLEA, chemiluminescent enzyme immunoassay; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay.
Stability of t-tau protein CLEA measurements in the control and test samples

One sample was injected in 10 replicates and measured twice daily at different time points, whereas the t-tau measurements were assessed over an additional 5-day period (Table 2). The SD values ranged from 10.1 to 31.7, indicating substantial fluctuations of >1,000 pg/mL in the t-tau protein CLEA measurements. Moreover, the CV ranged from 1.9 to 4.2%, suggesting consistent CV values below a threshold of 5% [19].

<table>
<thead>
<tr>
<th>Control samples</th>
<th>Test samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>aliquot 1</td>
<td>aliquot 2</td>
</tr>
<tr>
<td>Day 1-1</td>
<td>302</td>
</tr>
<tr>
<td>Day 1-2</td>
<td>307</td>
</tr>
<tr>
<td>Day 2-1</td>
<td>315</td>
</tr>
<tr>
<td>Day 2-2</td>
<td>320</td>
</tr>
<tr>
<td>Day 3-1</td>
<td>312</td>
</tr>
<tr>
<td>Day 3-2</td>
<td>321</td>
</tr>
<tr>
<td>Day 4-1</td>
<td>333</td>
</tr>
<tr>
<td>Day 4-2</td>
<td>333</td>
</tr>
<tr>
<td>Day 5-1</td>
<td>308</td>
</tr>
<tr>
<td>Day 5-2</td>
<td>327</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
</tr>
<tr>
<td>Min</td>
<td>302</td>
</tr>
<tr>
<td>Max</td>
<td>333</td>
</tr>
<tr>
<td>Mean</td>
<td>317.8</td>
</tr>
<tr>
<td>SD</td>
<td>10.3</td>
</tr>
<tr>
<td>CV (%)</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Table 2. Stability of the t-tau protein CLEA measurements

Control samples: The content of T-tau protein in all samples was detected by ELISA.

Test samples: The content of T-tau protein in all samples was detected by CLEA.

Abbreviations: SD, standard deviation; CLEA, chemiluminescent enzyme immunoassay; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay.

Storage stability of CSF samples
The 500-µL CSF samples from all the patients were divided into five 100-µL aliquots. T-tau protein levels declined after 2 days of storage at room temperature, with an approximate deactivation rate of 6-9% within a week under the same conditions (Figure 1). T-tau protein concentrations remain relatively stable in samples stored at 4°C, showing only around a 3% decrease compared to those stored at 14°C. T-tau protein levels measured in samples stored at -20°C and -80°C remain nearly unchanged over a period of 14 days but demonstrate around a 5% decrease after storage for up to 29 days. Considering the results obtained from multiple samples with concentrations exceeding 1,000 pg/mL, we inferred that any variations in the t-tau protein measurements in samples stored at -20°C and -80°C would fall within the margin of error (Figure 1).

Reproducibility of the results with t-tau protein dilution in CSF samples

We assessed the reproducibility of the results by diluting the t-tau protein samples, as well as by diluting the t-tau protein in the CSF samples, using various solutions to achieve a 50% reduction in the t-tau protein concentration (Table 3-a and b). Most dilution ratios ranged from 43 to 49%, with the dilution ratio resulting from deionised water being the closest to 50%. Although deionised water is considered a better, yet not optimal, dilution solution, we serially diluted the CSF samples multiple times using deionised water (Table 3). These findings suggest that as the data become more diluted, they tend to exhibit smoother, less distinct patterns (Table 3). In conclusion, serial dilutions are unsuitable for determining t-tau protein levels using CLEA.

Table 3a. Reproducibility of t-tau protein measurements on diluting the CSF samples. Results on diluting the tau protein sample by 1/2 dilution using three solutions (PBS, pure water, and the dissolving solution contained in the kit)

<table>
<thead>
<tr>
<th>Total tau protein measured using CLEA without dilution</th>
<th>1/2 dilution with PBS</th>
<th>1/2 dilution with pure water</th>
<th>1/2 dilution with dissolving solution contained in the kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 533</td>
<td>240 (0.450)</td>
<td>250 (0.469)</td>
<td>243 (0.456)</td>
</tr>
<tr>
<td>#2 628</td>
<td>295 (0.470)</td>
<td>301 (0.479)</td>
<td>308 (0.490)</td>
</tr>
<tr>
<td>#3 629</td>
<td>273 (0.434)</td>
<td>289 (0.459)</td>
<td>287 (0.456)</td>
</tr>
</tbody>
</table>

The ratio of tau protein measured at different dilutions with the undiluted solution set to 1 is included within parenthesis.

Ratio of tau protein measured at 1/2 dilution when the undiluted solution was set to 1

Abbreviations: PBS, phosphate-buffered saline; t-tau, total tau; CLEA, chemiluminescent enzyme immunoassay; CSF, cerebrospinal fluid
Table 3b. Results of stage dilution of t-tau protein in CSF samples using pure water

<table>
<thead>
<tr>
<th></th>
<th>Total tau protein measured using CLEA without dilution</th>
<th>1/2 dilution with pure water</th>
<th>1/4 dilution with pure water</th>
<th>1/8 dilution with pure water</th>
</tr>
</thead>
<tbody>
<tr>
<td>#4</td>
<td>1806</td>
<td>875 (0.484)</td>
<td>416 (0.230)</td>
<td>177 (0.098)</td>
</tr>
<tr>
<td>#5</td>
<td>1826</td>
<td>892 (0.488)</td>
<td>419 (0.229)</td>
<td>187 (0.102)</td>
</tr>
<tr>
<td>#6</td>
<td>1876</td>
<td>915 (0.484)</td>
<td>435 (0.232)</td>
<td>187 (0.100)</td>
</tr>
</tbody>
</table>

The ratio of tau protein measured at different dilutions with the undiluted solution set to 1 is included within parenthesis.

Ratio of tau protein measured at different dilutions with the undiluted solution set to 1

Abbreviations: t-tau, total tau; CLEA, chemiluminescent enzyme immunoassay; CSF, cerebrospinal fluid

**Relationship between t-tau protein levels measured by ELISA and CLEA**

The t-tau protein levels measured by CLEA in all samples in which t-tau protein levels were measured using ELISA (>2,200 pg/mL) exceeded 2,000 pg/mL. In patients with HPD, the t-tau protein levels measured by CLEA were consistently >1,300 pg/mL; however, in patients without HPD, the levels were <1,300 pg/mL. A comparison between t-tau protein levels measured by CLEA and ELISA revealed that the values obtained through CLEA were higher across all identical samples. A strong correlation (Figure 2) \( R^2 = 0.9363 \) was observed between ELISA and CLEA results, as shown in Additional Table 1 (Additional File 1). Due to the large number of patients having t-tau protein levels >2,000 pg/mL when measured using CLEA and due to patients without HPD having a level of 1,011 pg/mL, it was not possible to determine a specific cutoff point using the ELISA threshold value set at 1300 pg/mL. Using the value (1,011 pg/mL) with the highest sensitivity in CLEA as a reference against the ELISA cutoff point (1300 pg/mL) yielded a corresponding CLEA value of approximately 936 pg/mL. To further validate this correlation statistically, an analysis of variance (ANOVA) of the data (See Additional Table 2, Additional File 1) was performed, which indicated p-values ranging from 0.785 to 0.859. All values were >0.05, suggesting no significant difference in sensitivity between ELISA and CLEA.

**Results of biomarker measurements in the CSF of all patients**

The sensitivity and specificity results of the biomarker measurements in all patients are presented in Table 4, and the detailed biomarker data for all patients are shown in Additional Table 1 (Additional File 1). A positive reaction was seen in both the first- and second-generation RT-QuIC assays in all patients with sporadic HPD, whereas a negative reaction was seen in both assays in all patients without HPD. All patients with Huntington's disease (HD) tested positive for the presence of 14-3-3 protein measured using western blotting and for t-tau protein measured using ELISA (1,300 pg/mL). Conversely, patients without sporadic HPD tested negative for 14-3-3 protein measured using western blotting and t-tau protein
measured using ELISA (<1,300 pg/mL). Both the sensitivity and specificity of t-tau protein measurements with CLEA were 100%.

Table 4. Sensitivity and specificity of biomarker measurements in all patients

<table>
<thead>
<tr>
<th></th>
<th>RT-QuIC assay</th>
<th>14-3-3 protein by western blotting</th>
<th>Total tau protein by ELISA</th>
<th>Total tau protein by CLEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st generation</td>
<td>2nd generation</td>
<td>(cutoff: 1,300 pg/mL)</td>
<td>(cutoff: 1,000 pg/mL)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>97.8%</td>
</tr>
</tbody>
</table>

Abbreviations: RT-QuIC, real-time quaking-induced conversion; ELISA, enzyme-linked immunosorbent assay; CLEA, chemiluminescent enzyme immunoassay

Discussion

This was a 10-year prospective study (2011–2020), which involved a biomarker analysis in CSF samples of a cohort of 3,000 patients with rapidly progressive neurodegenerative conditions.

The original sample was divided into six aliquots that were assayed simultaneously. These samples were analysed in triplicate in both the control and HPD cases. The SD values ranged from 10.7 to 26.4, indicating that there were fluctuations of >1000 pg/mL in the t-tau protein levels measured by CLEA. Conversely, the CV values ranged from 1.2 to 3.7%, suggesting that the CV remained constant at <4% (Table 1) (See Additional Figure 1, Additional File 1).

Concurrently, we conducted additional testing using Mauchly's sphericity test and performed a conditional operation of Mauchly's sphericity test using statistical methods. Upon verifying normality (See Additional Table 3, Additional File 1), the p values of all data were >0.05. Subsequently, a Mochelay sphericity test was performed (See Additional Table 3, Additional File 1). Owing to the violation of the spherical hypothesis with a Greenhouse-Gessler value of 0.398, an epsilon (ε) correction was required. Since epsilon (ε) was <0.75, we employed the Greenhouse-Gessler method. In the time period correlation comparison, the Greenhouse-Geissler P value was calculated as 0.182, with an observed significance level of P>0.05, indicating no difference in ELISA results between the experimental and control groups at different time points. In the time x group analysis, the calculated Greenhouse-Geissler P-value was 0.348 with an observed significance level of P>0.05 (See Additional Table 4, Additional File 1), suggesting no difference in ELISA detection results between the experimental and control groups at different time points. Furthermore, by examining the contour line (See Additional Figure 2, Additional File 1), it was possible to determine whether there was a noticeable interaction. Both the control and experimental
groups exhibited significant changes over time with consistent trends, as indicated by the parallel line segments. This outcome aligns well with previously computed results.

Therefore, we can conclude that the CLEA detection method demonstrated high accuracy in simultaneously measuring t-tau levels in the test and control samples during reproducibility experiments. T-tau protein measurements were performed twice daily in 10 consecutive samples, with each sample injected once. Additionally, t-tau protein levels were measured using the same method for an additional 5 days (Table 2). The SD values ranged from 10.1 to 31.7, indicating a fluctuation of >1,000 pg/mL in t-tau protein levels measured by CLEA. Moreover, the CV ranged from 1.9 to 4.2%, suggesting consistent CV values of <5 (See Additional Figure 3, Additional File 1).

Concurrently, we employed Mauchly’s sphericity test to perform time period comparisons, intergroup comparisons, and an analysis of interactions between time periods and groups. First, a map of outliers visually displayed the degree of offset (CV%). This illustration demonstrates the reproducibility of diurnal differences in CLEA detection accuracy between the experimental and control groups. We aimed to determine whether the accuracy of CLEA was affected by the interactions between time and group using Mauchly's sphericity test. Initially, data normality was verified using calculations (See Additional Table 5, Additional File 1), yielding a result of P>0.1, which aligned with the normal distribution assumption. Subsequently, variance homogeneity was assessed, and the results indicated that the fluctuation of F values at the 10 time points ranged from 0.457 to 1.103 (P ranging from 0.353 to 0.536). The quality of variance was satisfied at each time point.

The assumptions for employing Mauchly's sphericity test were satisfied, enabling us to use this test to assess the impact of the interaction between groups and time periods on the accuracy of CLEA. Consequently, Mauchly’s sphericity test was conducted, and the results are presented in Additional Table 6 (Additional File 1). It is worth noting that Wend0.000 indicates an inability to determine the P-value as well as noncompliance with sphericity assumptions. Therefore, a direct analysis using the uncorrected method was not applicable; instead, an epsilon (ε) correction was necessary. Since our calculated epsilon (ε) value of 0.294 fell below 0.75, we employed a Greenhouse-Geisser correction for testing purposes (See Additional Table 6, Additional File 1).

The resulting P-value from the Greenhouse-Geisser correction was 0.017, which is <0.05, signifying significant differences in reproducibility outcomes across different time points. On another note, it should be mentioned that the p-value for the time x group interaction was >0.05 (See Additional Table 7, Additional File 1), indicating no substantial association between time points and interactive factors among groups; hence, there was no significant relationship between reproducibility outcomes at different time points.

The pairwise comparison results also indicated no significant correlation between the two groups at any time point. With a p-value >0.05 at all time periods, there was no significant difference between groups 1 and 2 at any given time point (See Additional Table 8, Additional File 1). Based on these findings, we can
confidently conclude that CLEA detection accurately replicates diurnal variations in the experimental group compared with the control group.

The 500-µL CSF samples from all patients were divided into five 100-µL aliquots. T-tau protein levels were decreased after 2 days of storage at room temperature, with a deactivation rate of approximately 6-9% within 1 week under the same conditions (Figure 1). At 4°C, t-tau protein levels remained relatively stable, showing only around a 3% decrease compared to those stored at 14°C. T-tau protein levels in samples stored at -20°C and -80°C remained almost unchanged over a period of 14 days but showed a decline of approximately 5% after storage for up to 29 days. Considering the results obtained from multiple samples with concentrations >1000 pg/mL, it can be assumed that changes in the t-tau protein levels in samples stored at -20°C and -80°C fall within the margin of error (Figure 1). Statistical analysis of the experimental data using the SPSS software revealed F value fluctuations ranging between 0.085 and 0.344 and P fluctuation values ranging between 0.785 and 0.859 (Additional File 1). It has also been demonstrated that the CLEA method has certain advantages in terms of the storage stability of CSF samples.

We assessed the reproducibility of measurements in the samples by diluting the t-tau protein samples, as well as diluting the t-tau protein in the CSF samples, using various solutions to achieve a 50% reduction in the t-tau protein concentration (Table 3a and b). Although the ratio of the dilution solution was close to 0.5 (50%), the actual dilution ratio ranged from 43 to 49%, with the ratio obtained through deionised water being the closest to 50%. Although deionised water is considered a better option, it is not an optimal dilution solution. Consequently, serial dilutions of the CSF samples were performed multiple times using deionised water (Table 3a and b) (Additional Figure 4a and b; Additional File 1). These findings suggest that increasing the data dilution leads to increased smoothing effects. In summary, serial dilutions are unsuitable for determining t-tau protein levels using CLEA.

The effect of blood contamination in the CSF samples of patients with sporadic HPD on the CLEA response was studied. Contamination with red blood cells can potentially affect the CLEA response, particularly during routine clinical examinations, where the inadvertent introduction of approximately 1 to 10% of blood into the sampler during lumbar puncture may lead to contamination of CSF samples. Therefore, it is imperative to establish well-defined sample processing guidelines prior to analysis. To assess this, we generated mixed samples by adding 1% and 10% of the whole blood to the CSF samples and evaluated their protein levels. Overall, our experimental findings demonstrate that even in the presence of up to 10% of whole blood contamination, CLEA detection remains highly sensitive in testing CSF samples with a CV of <3%. Conversely, as the quantity of blood in CSF samples increases, the accuracy of the conventional RT-QuIC method diminishes. When the contamination level exceeds 1,250 blood cells/µL, the effectiveness of the RT-QuIC method declines [20] (Table 5).

Table 5. Effect of blood contamination on CLEA responses in CSF samples
Group: group 1 and group 2 were randomly extracted cerebrospinal samples, and the amount of t-tau protein in the sample was added to the sample using the CLEA detection technique.

Abbreviations: CLEA, chemiluminescent enzyme immunoassay; CSF, cerebrospinal fluid; SEM, standard error of the mean; SD, standard deviation; CV, coefficient of variation

The findings of this study support the feasibility of using the new CLEA technique to detect total tau protein in the CSF of patients with HPD. On comparing ELISA and CLEA, CLEA resulted in superior daily reproducibility, storage stability, and freeze-thaw performance, whereas ELISA performed better in dilution tests. Therefore, the development of novel CLEA dilution solutions for future clinical trials is imperative. The experimental evidence supporting these findings was obtained through the analysis of CSF samples from 90 randomly selected patients from a pool of 3,000 patients, demonstrating that the sensitivity and specificity of both ELISA and CLEA are 100%. Furthermore, a strong correlation coefficient was observed between ELISA and CLEA measurements ($R^2=0.9363$) (Figure 2).

We must acknowledge the limitations of our study. First, the relatively small sample size may restrict the generalisability of these findings. Second, the homogeneity within our sample, consisting solely of individuals from a single ethnic subgroup, could introduce demographic variability; however, we made efforts to mitigate this influence through mixed modelling. The determination of biomarkers in CSF has demonstrated significant practicality in predicting pathology. Rapid, appropriate, and sensitive detection could benefit serological research and decision-making. However, the standardisation and simplification of biomarker measurement methods remain fundamental challenges in clinical practice. The pre-analytical and analytical variability of biomarkers for diagnosing human prion disease and related
dementias impede their widespread use in routine and clinical settings, as well as the establishment of universally recognized thresholds. Nevertheless, a simplified method that does not require specialised equipment such as ELISA would facilitate its extensive utilization at any location.

**Conclusions**

Here we demonstrate the superiority of the new CLEA method in terms of automation, surpassing even our standard ELISA for T-tau and 14-3-3 protein detection within our study cohort. In conclusion, our findings underscore the robustness and clinical utility of CLEA for analysing CSF samples as a valuable diagnostic tool for HPDs, warranting its inclusion in the comprehensive evaluation of patients presenting with human prion disease.

**Abbreviations**

ALP: alkaline phosphatase

AUC: relative area under the curve

CI: confidence interval

CJD: Creutzfeldt-Jakob disease

CLEA: chemiluminescent enzyme immunoassay

CNS: central nervous system

CSF: cerebrospinal fluid

CV: coefficient of variation

Diff: difference

DNA: deoxyribonucleic acid

EEG: electroencephalogram

ELISA: enzyme-linked immunosorbent assay

FFI: fatal familial insomnia

gCJD: genetic CJD

HPD: human prion disease

NPV: negative predictive value
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PPV: positive predictive value
prion PrP: protein
PrPC: cellular prion protein
PrPSc: scrapie prion protein
Rcf: relative centrifugal force rel.
Ref: reference
Rfu: relative fluorescence units
ROC: receiver operating characteristic
Rpm: revolutions per minute
RT-QuIC: real-time quaking-induced conversion
sCJD: sporadic CJD
SD: standard deviation
SEM: Standard error of the mean

**Declarations**

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data were obtained from the patient’s attending physician and the Japan Prion Disease Surveillance Committee at the time of the investigation and pathological autopsy. Data sharing does not apply to this article because no datasets were generated in the current study.

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

Participate in the conception and design of research, collection of data, analysis and interpretation of data Manuscript writing statistical test calculation: KW and TN.

All authors have read and agreed to the published version of this manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

All patients or family members of patients participating in this research have understood and gave their informed consent to participate. The research was conducted in accordance with the Declaration of Helsinki and the "Ethical Guidelines for Medical Research Involving Human Subjects" issued by the Ministry of Health, Labor and Welfare. The Ethics Committee, Nagasaki University Graduate School of Biomedical Sciences, endorsed the study protocol (ID Nos. UMIN000038398, UMIN000016855, and UMIN000003301). The Ethics Committee, Nagasaki University Graduate School of Biomedical Sciences, approved the study protocol. All patients or family members of patients participating in this research have understood and agreed to the ethics committee documents for this research.

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References


**Figures**

**Figure 1**

Storage stability of CSF samples

The 500-uL CSF samples from all the patients are divided into five 100-uL aliquots. T-tau protein levels decline after 2 days of storage at room temperature, with an approximate deactivation rate of 6-9% within
a week under the same conditions. T-tau protein concentrations remain relatively stable in samples stored at 4°C, showing only around a 3% decrease compared to those stored at 14°C. T-tau protein levels measured in samples stored at -20°C and -80°C remain nearly unchanged over a period of 14 days but demonstrate around a 5% decrease after storage for up to 29 days. Considering the results obtained from multiple samples with concentrations exceeding 1,000 pg/mL, we infer that any variations in the t-tau protein measurements between samples stored at -20°C and -80°C fall within the margin of error. CSF, cerebrospinal fluid.

Figure 2

Relationship between t-tau protein levels measured using ELISA and CLEA

ROC analysis is performed using Lumipulse to investigate the diagnostic performance between CLEA and ELISA and evaluate the ROC analysis of the new automated method. According to the detection results, there is a strong correlation between CLEA and ELISA, indicating that both detection methods can effectively detect T-tau protein. CLEA, chemiluminescent enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.