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## Article

# Detection Limitations of Prion Seeding Activities in Blood Samples from Patients with Sporadic Prion Disease

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**Abstract:** Human prion diseases (HPDs) are fatal neurodegenerative disorders characterized by abnormal prion proteins (PrP<sup>Sc</sup>). Numerous techniques have been devised to detect prion seeding activity, each with limitations. Here, we developed a technology for in vitro amplification of abnormal prion proteins (PrP<sup>Sc</sup>), thereby enabling their sensitive detection. Using the real-time quaking-induced conversion (RT-QuIC) PrP<sup>Sc</sup> amplification assay, we could detect  $\geq 1$  fg of PrP<sup>Sc</sup> in diluted human prion disease brain homogenate. Although RT-QuIC assay is suitable for detecting prion seeding activity in a variety of specimens, its accuracy in whole blood, blood plasma, or blood-contaminated tissues is reduced. To circumvent this issue and move toward developing a blood test for prions, we introduced an immunoprecipitation step at the beginning of the RT-QuIC reaction. We refer to this enhanced method as “eQuIC”. eQuIC utilizes three types of human monoclonal antibodies, which significantly increased its sensitivity—making it approximately 1000 times more sensitive than the original RT-QuIC assay. Nevertheless, when we tested the blood (serum, plasma, and whole blood) of six patients with sporadic human prion disease using eQuIC, we were unable to detect prion activity. Therefore, we concluded that the detection of prion seeding activities in blood samples from patients with sporadic prion disease remains challenging; thus, alternative methods beyond RT-QuIC and eQuIC will be necessary for future research.

**Keywords:** abnormal prion proteins; eQuIC; sporadic prion disease; prion seeding activities; diagnostic techniques

## 1. Introduction

Human prion diseases (HPDs), or transmissible spongiform encephalopathies (TSEs), are fatal transmissible neurodegenerative diseases in humans [1] and are classified into sporadic, genetic, and acquired forms, the most common being the sporadic form of the Creutzfeldt–Jakob disease (sCJD). The annual incidence rate of sCJD is 1.4 per million people in Japan [2]. Genetic prion disease is characterized by causal mutations in the human prion protein gene (*PRNP*) or a relevant family history, including Gerstmann–Straussler–Scheinker disease, fatal familial insomnia (FFI), or genetic CJD (gCJD). The acquired forms are dura-associated CJD (dCJD) and variant CJD derived from bovine spongiform encephalopathy [3].

The infectious agent, or prion, of TSEs appears to be composed of an abnormal prion protein (PrP<sup>Sc</sup>). PrP<sup>Sc</sup> is formed post-translationally from the normal cellular prion protein (PrP<sup>C</sup>), which in

purified form can resemble amyloid fibrils. PrP<sup>Sc</sup> induces the polymerization and conformational conversion of PrP<sup>C</sup> into infectious PrP<sup>Sc</sup> or PrP<sup>Sc</sup>-like partially protease-resistant PrP forms in a variety of in vitro reactions [1].

In vitro techniques for amplifying PrP<sup>Sc</sup>, such as protein misfolding cyclic amplification (PMCA) [4], amyloid seeding assay (ASA) [5], and quaking-induced conversion (QuIC) [6], enable the highly sensitive detection of PrP<sup>Sc</sup>. QuIC assays use recombinant normal prion protein as a substrate to amplify minute quantities of PrP<sup>Sc</sup> with intermittent automated shaking, which can be performed more easily than the PMCA-associated sonication. We recently developed a novel in vitro amplification technology, designated as the “real-time quaking-induced conversion (RT-QuIC)” assay, which enables the detection of prion seeding activity in various organ tissues derived from patients with sCJD [7]. Previously, we had developed the RT-QuIC assay primarily for identifying minute quantities of abnormal PrP within tissue and body fluids. We demonstrated that the RT-QuIC assay is more sensitive than bioassays. Notably, the end-point RT-QuIC assay can be used to evaluate human prion seeding activity (PSA) in the brains of patients with HPDs [8]. Given the recent advancements in drug development for prion diseases and the initiation of clinical trials, there is a growing need to measure prion activity in non-invasive tissues, such as blood. However, detecting PSA in blood poses considerable challenges. Therefore, to circumvent this issue and move toward developing a blood test for prions, we introduced an immunoprecipitation step at the beginning of the RT-QuIC reaction. We further enhanced the sensitivity through the addition of an rPrP substrate replacement step during the RT-QuIC reaction, an approach previously shown to be helpful in PMCA reactions [4]. The combination of these two assay modifications resulted in an enhanced method that we termed “eQuIC” [9].

In a previous study, the target animals of the “eQuIC” method were mice and humans, but the target disease in humans was variant CJD [10]. Notably, the “eQuIC” method is capable of detecting prion seeding activity from blood samples of patients with variant CJD. However, the detection of prion seeding activity from the blood of patients with sporadic HPD (sHPD) could not be achieved. In this study, we aimed to improve the “eQuIC” method for detecting prion seeding activities in the blood samples of patients with sHPD.

2. Materials and Methods

2.1. Sample Collection and Preparation

For our analysis, we used endpoint RT-QuIC assays to examine brain samples obtained from three confirmed cases of sHPDs (specifically of the MM1 subtype according to the Parchi classification [2]; the cases correspond to patients #1, #2, and #3). We included two non-CJD brain specimens as negative controls. The brain tissue samples were homogenized in 10% (w/v) ice-cold phosphate-buffered saline supplemented with a protease inhibitor mixture (Roche, Mannheim, Germany) using a multi-bead shocker (Yasui Kikai, Osaka, Japan). Brain homogenates (BHs) (10% [w/v]) were serially diluted (10-fold) using a material solution containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.2 ng/mL BSA, and 0.05% glucose. Thereafter, the samples were centrifuged at 6000 rpm and room temperature (15–20 °C) for 2 min to clarify the supernatant. The clarified samples were stored at –80 °C until further use.

We collected blood samples (total blood, serum, and plasma) from patients #1–3 within one month of onset of disease (Table 1). The Ethics Committee, Nagasaki University Hospital, endorsed the study protocol (ID Nos. UMIN000038398, UMIN000016855, and UMIN000003301).

Table 1. Summary of patients with sporadic human prion disease.

| Patient number                      | 1      | 2      | 3    |
|-------------------------------------|--------|--------|------|
| Sex                                 | Female | Female | Male |
| Age at onset (years)                | 69     | 74     | 73   |
| Time from onset till death (months) | 27     | 4      | 16   |

| sCJD subtype based on Parchi's classification   | MM1          | MM1          | MM1          |
|---|--------------|--------------|--------------|
| log SD <sub>50</sub> /g tissue in brain homogenates based on the RT-QuIC assay (mean ± SD)                | 10.38 ± 0.18 | 10.25 ± 0.35 | 10.00 ± 0.00 |
| log SD <sub>50</sub> /g tissue in brain homogenates based on the second-generation QuIC assay (mean ± SD) | 10.50 ± 0.50 | 10.75 ± 1.15 | 10.60 ± 1.25 |
| log SD <sub>50</sub> /g tissue in brain homogenates based on the eQuIC assay (mean ± SD)                  | 12.75 ± 0.35 | 12.25 ± 0.18 | 12.50 ± 0.35 |

Abbreviations: sCJD, sporadic Creutzfeldt-Jakob Disease; SD<sub>50</sub>, median seed dilution that induces a positive response in half of the tested samples; RT-QuIC, real-time quaking-induced conversion; QuIC, quaking-induced conversion; eQuIC, enhanced quaking-induced conversion; SD, standard deviation.

2.2. RT-QuIC, Endpoint RT-QuIC, and Second-Generation RT-QuIC Assay

Analyses of the RT-QuIC and endpoint RT-QuIC assays were similar to the previously described methodology for unfixed brain RT-QuIC assays [7]. In brief, the RT-QuIC reaction mix consisted of 50 mM PIPES (pH 7.0), 500 mM NaCl, 10 μM thioflavin-T (ThT), 0.1 mM ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA), and recombinant human PrP (residues 23–231 with 129 M). Each well of a black 96-well plate with a clear bottom (Nunc 96 well; Sigma-Aldrich, St. Louis, MO, USA) was loaded with 90-μL aliquots of the reaction mix and 10 μL of brain homogenate. Four to eight replicates of each diluted sample were assessed, and PrP amyloid formation was monitored for 48 h. We calculated the 50% seeding dose (SD<sub>50</sub>) in unfixed brains, fixed brains, and formic acid-treated brains using the Spearman–Kärber method [8].

We used the RT-QuIC assay and defined a reaction as positive when the following three conditions were met: (1) the absorbance exhibited an initial rise within 150 cycles, maintaining a stable level after attaining the maximum absorbance; (2) the maximum absorbance was >6-fold higher than the initial absorbance; and (3) only recombinant protein was used to maintain the quality of the batch, provided that the prepared recombinant protein displayed an SD<sub>50</sub> ≥ 10<sup>7</sup>, as determined using the RT-QuIC assay in the MM1-type sCJD brain.

Alternatively, the second-generation RT-QuIC assay was performed as previously described [11]. The assay was repeated at least twice, and Spearman–Kärber analysis was used to estimate the SD<sub>50</sub>. The SD<sub>50</sub> was calculated using the following formula:  $x_p = 1 + \frac{1}{2d} - dgp$ , where  $x_p = 1$  represents the highest log dilution that generates all positive responses,  $d$  represents the log dilution factor,  $p$  denotes the proportion positive at a given dose, and  $gp$  denotes the sum of  $p$  values for  $x_p = 1$  and all higher dilutions [9].

After purification, rHuPrP was stored at –80 °C. Brain homogenates (BHs) (10% [w/v]) were serially diluted (10-fold) with a material solution containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.2 ng/mL BSA, and 0.05% glucose. Each well of a 96-well plate was loaded with the rHuPrP suspended in 90 μL of RT-QuIC buffer (500 mM NaCl, 50 mM PIPES (pH 7.0), 10 μM ThT, and 1 mM EDTA), and mixed with 10 μL of the brain samples or blood samples. The assay was monitored for 40 h. Four to eight replicates of each diluted sample were measured. The SD<sub>50</sub> was calculated using the Spearman–Kärber method. The same organs derived from two healthy individuals were used as negative controls in all RT-QuIC assay experiments using eight wells.

2.3. Antibody (3F4, 3S9, and 2H9) Coating of Magnetic Beads

3F4 antibody is already the most used antibody by many prion researchers, and we collaborated with Hiroshima University to create two antibodies (3S9, and 2H9) to use in this experiment. These two antibodies recognize the C-terminus of the PRNP gene region 110–231. The antibodies (3F4, 3S9,

and 2H9) were identified in the brains of the patients with sHPD. We outsourced the general procedure of attaching the antibodies to magnetic beads to Tamagawa Seiki Kaihatsu Shikkai Co (Nagano prefecture, Chuubu area, Japan). Mock control beads were prepared as described for the 3F4, 3S9, and 2H9 antibody-coated beads, but without the addition of these antibodies.

#### 2.4. Preparation of MagnaBind Beads

MagnaBind beads (Pierce, Rockford, IL, USA) were vortexed for 30 s, and  $1.2 \times 10^8$  beads were transferred to a fresh tube. The beads were rinsed twice with 500  $\mu$ L of 0.5% Triton X-100 in PBS and resuspended in their initial volume using assay buffer (Tris-buffered saline, 1% Triton X-100, and 1% Tween 20).

#### 2.5. eQuIC Assay on Blood (Serum, Plasma, and Total Blood) and Brain Homogenates from Patients with sHPD

The antibody-coated beads, mock beads, and Magna Bind beads were briefly vortexed, and  $2 \times 10^7$  beads were transferred to a fresh tube. A volume of 500  $\mu$ L of human blood (serum, plasma, and total blood) was added to the beads. Following 2 min of incubation on the magnet, the storage (coating) buffer was discarded, and 500  $\mu$ L of immunoprecipitation buffer (Prionics) was added. Thereafter, 500  $\mu$ L of BH-spiked human plasma or 500  $\mu$ L of plasma or the brain homogenate from patients with HPD was added to the beads. The samples were incubated with “end-over-end” rotation at room temperature or 37 °C overnight (ON). Subsequently, the samples were incubated on the magnet for 2 min, the plasma-buffer mix was discarded, and beads were washed twice with 500  $\mu$ L of wash buffer. All beads were then resuspended in 10  $\mu$ L of PBS and used immediately.

### 3. Results

#### 3.1. RT-QuIC and Endpoint RT-QuIC, eQuIC and Second-Generation RT-QuIC Assays in Brain Homogenate from HPD Patients

Three patients with HPD were designated as the MM1 subtype based on Parchi classification. The log  $SD_{50}$  obtained from the RT-QuIC assay for these three patients (patients #1, #2, and #3) were  $10.38 \pm 0.18$ ,  $10.25 \pm 0.35$ , and  $10.00 \pm 0.00$ , respectively. Similarly, using the second-generation QuIC assay, the log  $SD_{50}$  values for the patients were  $10.50 \pm 0.50$ ,  $10.75 \pm 1.15$ , and  $10.60 \pm 1.25$ , respectively. The newly developed eQuIC assay in the HPD patients yielded  $SD_{50}$  values of  $12.75 \pm 0.35$ ,  $12.25 \pm 0.18$ , and  $12.50 \pm 0.35$ , respectively (Table 1). Upon comparing the log  $SD_{50}$  values between the eQuIC and RT-QuIC methods, our results indicate that the eQuIC assay displays an approximate 2–2.5-fold higher sensitivity than the RT-QuIC method. Considering that this sensitivity increment translates to an approximate 100–300-fold enhancement in sensitivity, our method might have the potential to detect as little as  $\geq 10$  ag of PrP<sup>Sc</sup> in brain homogenates derived from patients with HPD.

Given the possibility that this may be attributable to differences in the sensitivity of recombinant proteins, we investigated the differences in recombinant proteins between humans and hamsters. We also examined the performance of the second-generation RT-QuIC assay using recombinant proteins in hamsters (Supplementary Material [14,15] and Table 1). Our results suggest that the RT-QuIC and the second-generation RT-QuIC assays achieved similar  $SD_{50}$  values for recombinant proteins in hamsters (Supplementary Table S1).

#### 3.2. eQuIC Assay on Blood Samples from HPD Patients

We performed approximately 1,000 different eQuIC assays on blood (plasma, serum, and total blood) samples from patients with HPD; however, we were unable to detect PSAs in the blood samples using the eQuIC assay (Table 2).



**Table 2.** Summary of sporadic human prion disease patient blood (serum, plasma, and total blood) analyzed using the eQuIC assay.

| Patient number   | 1            | 2            | 3            |
|--|--------------|--------------|--------------|
| log SD <sub>50</sub> /g tissue in brain homogenates based on the eQuIC assay (mean ± SD) | 12.75 ± 0.35 | 12.50 ± 0.18 | 12.15 ± 0.35 |
| log SD <sub>50</sub> /g tissue in blood based on the eQuIC assay                         | N.D.         | N.D.         | N.D.         |

Abbreviations: SD<sub>50</sub>, median seed dilution that induces a positive response in half of the tested samples; eQuIC, enhanced quaking-induced conversion; N.D., not detected.

4. Discussion

We recently developed a novel in vitro amplification method, the eQuIC assay, which enabled the detection of ≥10 ag of PrP<sup>Sc</sup> from diluted brain homogenates from patients with sHPD. The eQuIC assay was approximately 100 times more sensitive than the RT-QuIC assay at detecting PrP<sup>Sc</sup> from brain homogenates of patients with sHPD.

Furthermore, Orrú et al. highlighted the potential of eQuIC for routinely detecting minute quantities of prion in tissues, fluids, or environmental samples [9,10]. They utilized the eQuIC assay to detect prions in the plasma of patients with variant CJD and demonstrated that eQuIC could effectively distinguish between plasma samples from infected and uninfected hamsters and sheep with scrapie.

Furthermore, evidence points to the presence of infectious agents in the erythrocytes, leukocytes, and plasma of both variant and sporadic CJD patients [12,13]. The measured infectivity levels were comparable to those reported in various prion disease animal models [8].

However, our attempts to detect PSAs in the plasma, whole blood, and serum from sHPD patients were unsuccessful. We propose three potential reasons for this: 1) The concentration of PrP<sup>Sc</sup> in the blood of patients with sHPD may have been minuscule, rendering its detection with our current technology unfeasible; 2) There might be no detectable PSAs in the blood of these patients; 3) Although our eQuIC assay was shown to be highly sensitive for brain tissue detection, the antibodies used might not have been well-suited for blood-based detection. Based on insights from past research, we consider all three hypotheses to account for our findings. However, we believe the combined likelihood of the first and second hypotheses to be the most plausible explanation. Hence, we recognize the importance of exploring alternative detection methods beyond eQuIC for measuring prion activity from blood samples of patients with sHPD.

In conclusion, although we successfully developed a novel method (eQuIC) that is approximately 100 times more sensitive than previously used amplification methods, its application for blood-based PSA detection remains inconclusive. Therefore, new methods must be explored to detect prion activity from the blood of patients with prion diseases.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Methods: Production of the Hamster Truncated Recombinant Prion Protein (rHaTrPrP) 90-231; Supplementary Material and protocols for the second-generation QuIC assay.

**Author Contributions:** Neuropathological analysis and Parchi’s classification, YI; supply for blood samples, K.S. and T.N.; production of antibodies, H.H.; writing—preparation of the original draft, K.S. and T.N.; writing—preparation of the supplementary material, K.S. and T.N. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** We obtained consent from all authors for publication.

**Data Availability Statement:** Data sharing does not apply to this article, as no datasets were generated during the current study.

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