Video Article

Real-time Quaking-induced Conversion Assay for Detection of CWD Prions in Fecal Material

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Abstract

The RT-QuIC technique is a sensitive *in vitro* cell-free prion amplification assay based mainly on the seeded misfolding and aggregation of recombinant prion protein (PrP) substrate using prion seeds as a template for the conversion. RT-QuIC is a novel high-throughput technique which is analogous to real-time polymerase chain reaction (PCR). Detection of amyloid fibril growth is based on the dye Thioflavin T, which fluoresces upon specific interaction with β-sheet rich proteins. Thus, amyloid formation can be detected in real time. We attempted to develop a reliable non-invasive screening test to detect chronic wasting disease (CWD) prions in fecal extract. Here, we have specifically adapted the RT-QuIC technique to reveal PrP^{Sc} seeding activity in feces of CWD infected cervids. Initially, the seeding activity of the fecal extracts we prepared was relatively low in RT-QuIC, possibly due to potential assay inhibitors in the fecal material. To improve seeding activity of feces extracts and remove potential assay inhibitors, we homogenized the fecal samples in a buffer containing detergents and protease inhibitors. We also submitted the samples to different methodologies to concentrate PrP^{Sc} on the basis of protein precipitation using sodium phosphotungstic acid, and centrifugal force. Finally, the feces extracts were tested by optimized RT-QuIC which included substrate replacement in the protocol to improve the sensitivity of detection. Thus, we established a protocol for sensitive detection of CWD prion seeding activity in feces of pre-clinical and clinical cervids by RT-QuIC, which can be a practical tool for non-invasive CWD diagnosis.

Video Link

The video component of this article can be found at https://www.jove.com/video/56373/

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSE) are neurodegenerative disorders including Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, and chronic wasting disease (CWD) in cervids [1,2]. TSEs are characterized by distinctive spongiform appearance and loss of neurons in the brain. According to the "protein only" hypothesis, prions are mainly composed of PrP^{Sc} ('Sc' for scrapie) [3], a misfolded isoform of the host-encoded cellular prion protein, PrP^{C}. PrP^{Sc} results from the conversion of PrP^{C} into a conformation enriched in β-sheets [4,5,6] which can act as a seed to bind and convert other PrP^{C} molecules. The newly generated PrP^{Sc} molecules are incorporated into a growing polymer [7,8] which breaks into smaller oligomers, resulting in higher numbers of infectious nuclei. PrP^{Sc} is prone to aggregation and is partially resistant to proteases [9,10].

CWD affects wild and farmed elk (*Cervus canadensis*), mule deer (*Odocoileus hemionus*), white-tailed deer (WTD; *Odocoileus virginianus*), moose (*Alces alces*), and reindeer (*Rangifer tarandus tarandus*) [11,12,13]. It is considered the most contagious prion disease with horizontal transmission favored by cervid interactions and environmental persistence of infectivity [14,15]. Unlike other prion diseases where PrP^{Sc} accumulation and infectivity are confined to the brain, in CWD these are also found in peripheral tissues and body fluids e.g. saliva, urine, and feces [16,17,18].

Immunohistochemistry is considered the gold standard for CWD diagnosis to detect PrP^{Sc} distribution and spongiform lesions [19,20]. ELISA and in more rare cases, western blot are also used for CWD diagnostics. Thus, current prion disease diagnosis is mainly based on detecting prions in post-mortem tissues. Ante-mortem diagnosis for CWD is available by taking tonsils or recto-anal mucosa-associated lymphoid tissue (RAMALT) biopsies; however, this procedure is invasive and requires the capture of the animals. Thus, the use of easily accessible specimens, such as urine and feces, would be a practical way for CWD prion detection. However, those excreta harbor relatively low concentrations of prions below the detection limit of current diagnostic methods. Consequently, a more sensitive and high throughput diagnostic tool is needed. *In vitro* conversion systems, such as protein misfolding cyclic amplification assay (PMCA) [21], amyloid seeding assay, and real-time quaking-induced conversion (RT-QuIC) assay [22,23,24] are very powerful tools to exploit the self-propagating ability of PrP^{Sc} to mimic *in vitro* the prion conversion process and thereby amplify the presence of minute amounts of PrP^{Sc} to detectable levels [25,26]. The RT-QuIC method, however,

takes advantage of the fact that the conversion product enriched in β-sheet secondary structure can specifically bind thioflavin T (Th-T). Therefore, recombinant PrP (rPrP) upon seeded conversion grows into amyloid fibrils which bind Th-T and thus can be detected in real time by measuring the fluorescence of Th-T expressed as relative fluorescence units (RFU) over time. Once monitored, the RFU can be used to evaluate relative seeding activities, and quantitative parameters such as the lag phase. The lag phase represents the time (h) required to reach the threshold, during which rPrP conversion at the early stage of the reaction is below the detection limit of Th-T fluorescence. The end of the apparent lag phase, concomitant to the formation of a sufficient amyloid nucleus (nucleation/elongation), occurs when the Th-T fluorescence exceeds the threshold level and becomes positive. The growth of amyloid fibrils can be detected in real time and the initial PrP^{Sc} or seeding activity contained in the sample is amplified by segmentation which generates more seeds. These seeds in turn induce a rapid exponential phase of amyloid fiber growth.

Because this assay is able to detect as low as 1 fg of PrP^{Sc} [24], the high sensitivity qualifies this technique to achieve ante mortem or non-invasive diagnosis by detecting PrP^{Sc} in various peripheral tissues, excreta or other kinds of specimen harboring low levels of infectivity. RT-QuIC definitely provides advantages over other assays for its reproducibility, practicality, rapidity (less than 50 h) and low costs compared to bioassays. It avoids the technical complexities such as sonication used in PMCA; also, it is performed in a tape-sealed microplate which minimizes the risk of aerosol contamination of each well. The multi-well format enables the analysis of up to 96 samples in the same experiment. To counter the recurrent problem of false positives and spontaneous conversion of rPrP in the *in vitro* conversion assays the implementation of a threshold (cut-off) in RT-QuIC is very useful. Indeed, based on the results of the negative control (average RFU of negative samples +5 SD [27]), a baseline is set up from which discrimination between positive and negative samples can be done. The use of four replicates for each sample can thus help to define a sample as positive when at least 50% of the replicates show a positive signal, *i.e.* cross the cut-off [28]. The homology between seed and substrate is not required in RT-QuIC, as e.g. in a previous study, hamster rPrP was found to be a more sensitive substrate compared to the homologous substrate in human PrP^{vCJD} seeded and sheep scrapie seeded reactions [29]. Hamster-sheep chimeric rPrP was also suggested to be a more well-suited substrate than human rPrP to detect human variant CJD prions [30]. Thus, the use of rPrP substrates from different species is very common in this assay. This assay has been successfully applied to several prion diseases, such as sporadic CJD [31,32,33], genetic prion diseases [34], BSE [35,36,37], scrapie [23,36], and CWD [38,39,40,41,42]. Studies using processed cerebrospinal fluid, whole blood, saliva, and urine as seeds in RT-QuIC were all successful to detect PrP^{Sc} [38,39,40,41,42]. To foster the detection ability in samples such as blood plasma that may contain inhibitors of amyloid formation, Orrú *et al*. (2011) developed a strategy to remove potential inhibitors of amyloid formation by combing PrP^{Sc} immunoprecipitation (IP) step and RT-QuIC, named "enhanced QuIC" assay (eQuIC). In addition, a substrate replacement step was employed after ~24 h of reaction time in order to improve the sensitivity. Ultimately, as low as 1 ag of PrP^{Sc} was detectable by eQuIC [30].

In order to purify feces extracts and remove possible assay inhibitors in feces, fecal samples collected at preclinical and clinical stages from elk upon experimental oral infection were homogenized in buffer containing detergents and protease inhibitors. The feces extracts were further submitted to different methodologies to concentrate PrP^{Sc} in the samples utilizing protein precipitation via sodium phosphotungstic acid (NaPTA) precipitation. The NaPTA precipitation method, first described by Safar *et al.*[43], is used to concentrate PrP^{Sc} in test samples. The incubation of NaPTA with the sample results in preferential precipitation of PrP^{Sc} rather than PrP^{C}. However, the molecular mechanism is still unclear. This step also helped containing and preventing the spontaneous conversion of rPrP, which is observed in some cases. Finally, the feces extracts were tested by optimized RT-QuIC using mouse rPrP (aa 23-231) as a substrate and including substrate replacement in the protocol to improve the sensitivity of detection.

The results here demonstrate that this improved method can detect very low concentrations of CWD prions and increases the sensitivity of detection and specificity in fecal samples compared to a protocol without NaPTA precipitation and substrate replacement. This method potentially can be applied to other tissues and body fluids and can be of great use for CWD surveillance in wild and captive cervids.

Protocol

1. RT-QuIC Using Fecal Material

1. **Preparation of cervid feces extracts**
    1. Make fecal homogenate by adding 1 g of fecal material to 10 mL of feces extract buffer (20 mM sodium phosphate, pH 7.1, 130 mM NaCl, 0.05% Tween 20, 1 mM PMSF and 1x complete protease inhibitors, EDTA-free) to give a final concentration of 10% (w/v). The homogenization buffer can be prepared prior to the utilization and stored at -20 °C.
    2. Homogenize fecal pellets (1 g) and buffer (10 mL) in tubes (e.g., gentleMACS M tubes) using a dissociator with a pre-set program from the manufacturer for proteins for 1 min at room temperature. Repeat this step two to three times until the fecal samples are completely homogenized in the buffer.
    3. Seal the tubes with parafilm and place them onto a rocking platform or rotary shaker for a 1 h incubation at room temperature.
    4. Centrifuge the tubes at 18,000 x g for 5 min at room temperature.
    5. Collect the supernatants which contain the protein extracts and aliquot them into 1.5 mL tubes. Aliquots can be stored at -80 °C for further applications.

2. **Concentration of PrP^{Sc} in feces extracts**
   NOTE: In order to concentrate CWD prions in the feces extract samples, thereby improving the sensitivity of detection by RT-QuIC, a protein precipitation step is added to the protocol.
    1. NaPTA precipitation method
    2. Add 250 µL of 10% (w/v) of N-lauryl sarcosinate (sarkosyl) to 1 mL of fecal protein extract to give a final concentration of sarkosyl of 2% (v/v).
    3. Seal the tubes which contain the samples with parafilm and incubate at 37 °C for 30 min under constant shaking at 1,400 rpm in a thermomixer.
    4. Adjust the mix of fecal protein extract and sarkosyl with a stock solution containing 10% (w/v) of sodium phosphotungstic acid and 170 mM of magnesium chloride, pH 7.4 to obtain a final concentration of 0.3% (w/v) sodium phosphotungstic acid in the samples.
    5. Incubate the samples at 37 °C for 2 h with constant shaking at 400 rpm.

6. Centrifuge the samples at 14 °C for 30 min at 15,800 x g. Remove the supernatants carefully.
7. Wash the pellets by resuspending them in wash buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5% Triton-X 100, 10 mM EDTA, 0.5% sodium deoxycholate (w/v), and 0.1% sarkosyl (w/v)).
8. Centrifuge the samples at 14 °C for 15 min at 15,800 x g. Remove the supernatants carefully.
9. Resuspend the pellets in 100 µL (1/10 of the original volume of the fecal protein extract) of RT-QuIC dilution buffer.
   NOTE: RT-QuIC dilution buffer (20 mM sodium phosphate, pH 6.9, 130 mM NaCl, 0.1% SDS (w/v) and 1 X N2 supplement) is prepared prior to the utilization. A total volume of 10 mL is filtered through a 0.2 µm acrodisc syringe filter using a syringe and then stored at -20 °C until use.
10. Store the NaPTA treated samples at -20 °C until utilizing them in RT-QuIC assay.

3. **RT-QuIC assay**
   1. Prepare a fresh 10 mM Th-T stock solution (0.032 g of Th-T in 10 mL of double distilled H_2O).
   2. Make a dilution of 1:10 of the Th-T stock solution in double distilled H_2O to make a final concentration of Th-T of 1 mM the filter it through a 0.2 µm acrodisc syringe filter using a syringe.
   3. Thaw mouse recombinant PrP (rPrP, aa 23-231) substrate (10 µg/mL per RT-QuIC reaction) stored at -80 °C on ice.
   4. Load 500 µL of rPrP substrate at a time into 100 kDa size exclusion filter microtubes and centrifuge at 14,000 x g for 5 min at room temperature (one tube/filter can be used up to two times).
   5. Transfer the eluted substrate into a sterile 1.5 mL tube and keep it at 4 °C until use.
   6. Thaw RT-QuIC dilution buffer stored at -20 °C.
   7. Make dilutions of the seed (fecal protein extract) in RT-QuIC dilution buffer:
      Undiluted sample: use the fecal protein extract undiluted
      Diluted 2 x 10^{-1}
      Diluted 2 x 10^{-2}
      Diluted 2 x 10^{-3}
   8. Prepare stock solutions for RT-QuIC reaction mixture:
      Phosphate-buffered saline (PBS): 5X
      NaCl: 2 M stock solution of NaCl prepared in double distilled H_2O.
      EDTA: 100 mM stock solution of EDTA prepared in double distilled H_2O.
   9. Prior to utilization of all the solutions (step 1.3.8), filter each solution separately through a 0.2 µm acrodisc syringe filter using a syringe and keep them sterile at room temperature.
   10. Prepare a total volume of RT-QuIC reaction mixture according to the number of samples (98 µL volume of reaction per sample and four replicates per sample) to be used plus 10% of this volume to make sure to have enough mixture for all the reactions.
       1. Use a 15 mL tube to prepare the RT-QuIC reaction mixture (20 mM sodium phosphate, pH 6.9, 300 mM NaCl, 1 mM EDTA, 10 µM Th-T, 10 µg/mL rPrP substrate, and double distilled water to adjust the rPrP final concentration in the reaction) using the stock solutions.
       2. Mix all the solutions well by pipetting up and down using a pipette with a filter tip. Avoid as much as possible the use of vortex.
       3. Pour the mixture into a 50 mL disposable pipetting reservoir.
       4. Use a multichannel pipette to load 98 µL of RT-QuIC reaction mixture into each well of a 96-well optical bottom plate.
   11. Add to each RT-QuIC reaction 2 µL of undiluted or 10-fold serially diluted fecal protein extract. Test each sample in four replicates.
       NOTE: In each experiment, serial dilutions of fecal samples (ranging from undiluted to 2 x 10^{-3}) from CWD-negative and verified CWD-positive animals are used as negative and positive controls, respectively.
   12. Seal the plate with a sealing tape before incubating in a plate reader at 42 °C for 25 h with repeated cycles of 1 min double orbital shaking (700 rpm) and 1 min resting throughout the incubation.
   13. Define fluorescence measurement settings:
       Excitation: 450 nm
       Emission: 480 nm
       Bottom read, number of flashes: 20
       Manual gain: 1000 (depends on the machine)
       Integration time: 20 µs
   14. Remove the plate from the plate reader at the end of the 25 h.
   15. Spin down the plate at 3,000 x g for 3 min to avoid potential contamination between the wells.
   16. Remove the seal from the plate.
   17. Prepare a new 96-well plate by adding 90 µL of RT-QuIC reaction mixture containing fresh substrate and fresh fluorescence dye Th-T as described in section 1.3.1 to 1.3.6.
   18. Transfer 10 µL from each well of the first plate to the newly prepared 96 well plate.
   19. Seal the new plate and continue the RT-QuIC assay for additional 50 h following the steps described in step 1.3.13. This additional step of 50 h will make the total reaction time of 75 h.
       NOTE: All the procedures of RT-QuIC assay are done under biosafety cabinet.
   20. Collect the data.
       1. Read and document the Th-T fluorescence signal of each well every 15 min.
       2. Collect data of total cycles using Data Analysis software and transfer into a spreadsheet with the average of quadruplicate reactions.
       3. Plot the averages of data. The x-axis corresponds to the reaction time of RT-QuIC (h) and the y-axis corresponds to the relative fluorescence units (RFU). Each curve corresponds to a dilution of a known sample.
       4. In each experiment, demarcate a threshold by calculating the highest mean values of the negative control plus 5 standard deviations as described in current published literature [41].

NOTE: All replicates which crossed the defined threshold are considered positive. If at least two out of four replicates cross the threshold, the sample is then considered positive.

2. Purification of Recombinant Prion Protein (rPrP)

1. **Growth of bacterial stock and expression of rPrP**
   NOTE: The *Escherichia coli* (*E. coli*) strain Rosetta (DE3) transformed with vector pET-24 encoding the mouse PrP (residues 23-231) was used to prepare the rPrP.
   1. Thaw glycerol stock of Rosetta (DE3) transformed with pEt-24 mPrP(23-231) on ice.
   2. Streak LB (Luria Bertani) plates with a final concentration of kanamycin of 50 µg/mL and incubate overnight in a 37 °C incubator. Make sure that the plates are upside down to avoid humidity condensation in the solid media containing the bacteria. Prepare two plates to make sure to have growth in at least one of the two plates.
   3. Prepare 1 L of LB and autoclave it to make it sterile.
   4. Supplement 1 L of sterile LB media with 1 mL of kanamycin (50 mg/mL) and 1 mL of chloramphenicol (34 mg/mL).
   5. Pick one colony of each plate by using a disposable inoculation loop to inoculate 3 mL of LB medium containing kanamycin and chloramphenicol.
   6. Place the mini-cultures in an incubator at 37 °C with constant shaking at 225 rpm for 5-6 h.
   7. Add the mini-cultures to the rest of the original 1 l of LB media together with Express Autoinduction System 1 for induction of protein expression.
   8. Place the culture in 2 L flask, or larger, in an incubator at 37 °C with constant shaking at 200 rpm for 20-24 h.
   9. Split the 1 L culture into 4 x 250 mL centrifuge tubes.
   10. Spin down the tubes containing the culture at 3,750 x g for 20 min at room temperature.
   11. Discard the supernatant and collect the bacterial pellets in 50 mL centrifuge tubes, then freeze them at -80 °C until further processing. The resulting pellets have weighed 3 to 4 g for a good protein yield.

2. **Preparation of inclusion body**
   1. Thaw the frozen pellet (3 to 4 g) at 37 °C for few minutes.
   2. Freeze the pellet one more time for 10 min at -80 °C and thaw again. Repeat this step of freezing and thawing two more times.
   3. Resuspend the bacterial pellet in 1X *lysis reagent (e.g.* BugBuster Master Mix) by pipetting thoroughly. Use 5 mL of the reagent for 1 g of bacterial pellet. Make sure to completely homogenize the mix.
   4. Incubate the homogenate on a rocker for 20 min at room temperature.
   5. Centrifuge the homogenate at 16,000 x g for 20 min at room temperature.
   6. Discard the supernatants by carefully pouring it off.
   7. Homogenize the pellets in the same volume of 1X lysis reagent as used in the previous step.
   8. Incubate the homogenate for 15 min on a rocker at room temperature.
   9. Add a sufficient volume of 1:10 (0.1x) lysis reagent to get a total homogenate volume of 40 mL. Mix by inversion to make sure to homogenize well.
   10. Centrifuge the homogenate at 7,900 x g for 15 min at 4 °C.
   11. Discard the supernatant by carefully pouring it.
   12. Resuspend the pellet in 40 mL of 0.1x lysis reagent.
   13. Centrifuge the homogenate at 16,000 x g for 15 min at 4 °C.
   14. Discard the supernatant carefully by pouring it and store the pellet containing the inclusion bodies at -20 °C until further processing.

3. **Protein purification**
   1. Thaw the inclusion bodies at room temperature.
   2. Dissolve the thawed inclusion bodies in 14 mL of 8 M guanidine-HCl (38 g Guanidine hydrochloride in 0.1 M $NaPO_4$ pH 8.0 and fill the suspension with H_2O to 50 mL; do not adjust pH at final solution) to solubilize proteins.
   3. Make sure to homogenize well by pipetting up and down.
   4. Incubate the lysate on a rocker at room temperature for 50 min.
   5. Prepare 18 g of Ni-NTA resin beads (18 g for 3 to 4 g bacterial pellet); rinse them with 100 mL of water using vacuum and a 100 mL 0.22 µm bottle top filter.
      1. Place the 18 g of Ni-NTA resin beads in 50 mL tube and add sufficient volume of denaturing buffer (100 mM sodium phosphate, 10 mM Tris, pH 8.0, 6 M guanidine hydrochloride, pH 8) to make the final volume up to 50 mL.
      2. Equilibrate the beads in the denaturing buffer for 50 min on a rocker at room temperature.
   6. Centrifuge the inclusion body lysate at 16,000 x g for 5 min to remove insoluble debris.
   7. Add the supernatant to the equilibrated beads and discard the pellets.
   8. Put the mix on a rocker for 40 min at room temperature to allow protein binding to Ni-NTA resin.
      NOTE: Nickel chelate beads are used to purify PrP by its nickel affinity. The histidine-rich N-terminal region of PrP renders the affinity to nickel(II), which allows the protein to bind to nickel ions without any his-tag.
   9. Wash FPLC with water to clean out both lines (A and B).
   10. Run denaturing buffer (100 mM sodium phosphate, 10 mM Tris, pH 8.0, 6 M guanidine hydrochloride, pH 8) through both lines (A and B) to prime the system (the column is not yet attached.)
   11. Load the resin onto column. Make sure to minimize air bubbles.
   12. Attach the column to the FPLC and run a linear gradient from 100% denaturing buffer A and 0% of refolding buffer B (100 mM sodium phosphate, 10 mM Tris, pH 8.0) at first to 0% denaturing buffer and 100% refolding buffer by the end. This gradual shift is run at a flow rate of 0.75 mL/min for 240 min and is needed for proper folding of PrP.

NOTE: During the chromatographic purification process, the denatured PrP is first slowly refolded on the resin by applying a linear gradient of refolding buffer to replace the denaturing buffer. This step also removes the chaotropic guanidine-HCl.

13. Make sure 100% refolding buffer continues to flow through the column for an additional 30 min at 0.75 mL/min.
14. Rinse line A with water followed by elution buffer (100 mM sodium phosphate, 10 mM Tris, 500 mM imidazole, pH 5.8) bypassing the column. This will clean out the denaturing buffer which has been replaced now by the elution buffer in the AKTA system.
15. Elute protein at 2 mL/min by running a linear gradient for 40 min from 100% refolding buffer B and 0% elution buffer A at first to 0% refolding buffer and 100% elution buffer.
    NOTE: The high concentration of imidazole in elution buffer will compete PrP's binding to the nickel(II). The primary protein peak should start to elute about 1/3 of the way through the gradient.
    1. Watch chromatogram for OD 280 nm to increase.
16. Collect only the tubes containing the protein at the center of the large peak.
    NOTE: The eluted fractions of 2 mL each are collected into 15 mL tubes.
17. Dilute the protein contained in the tubes immediately with about 1/3 volume (1 mL) of dialysis buffer (10 mM sodium phosphate, pH 5.8).
18. Immediately place the tubes on ice.
19. Pool the eluted protein contained in the tubes.
20. Poor the protein into Dialysis Cassettes (molecular weight cut-off 10 kDa).
21. Put the cassettes in pre-chilled dialysis buffer (3.6 L) for 2 h at 4 °C, then transfer the cassettes into a fresh dialysis buffer (3.6 L) for overnight. Make sure that the dialysis buffer is under constant agitation.
22. Filter protein after dialysis through a 0.2 µm acrodisc syringe filter using a syringe.
23. Measure the protein concentration using a BCA protein assay kit.
24. Concentrate or dilute if necessary to adjust the protein concentration to 0.3 mg/mL.
    NOTE: Confirm purity by SDS-PAGE and Coomassie Blue staining.
25. Make 1 mL aliquots of protein in microcentrifuge tubes and immediately store at -80 °C until further use as described in the RT-QuIC protocol.

Representative Results

The CWD fecal extracts prepared at 10% (w/v) were able to seed RT-QuIC reaction, yet the sensitivity of detection was low [27]. Using a specific buffer for fecal homogenization was a critical step to avoid high background fluorescence in RT-QuIC reactions concomitant to the use of mouse rPrP substrate rather than deer rPrP which allowed to get more specific results [27]. The addition of NaPTA precipitation reduced the spontaneous conversion of rPrP in RT-QuIC (**Figure 1**) without inhibiting amplification of the seeding activity of the reactions (**Figure 2**). Although better amplification was observed upon NaPTA treatment, resulting fluorescence signals of CWD-positive fecal samples were still low (**Figure 1**). In addition, the prion amplification of some samples reached a constant plateau at low fluorescence levels. This indicates the saturation of reactions, which can be due to the degradation/denaturation of rPrP or the formation of off-pathway aggregates which consume the rPrP pool [30]. To further improve the amplification of prions and enhance the sensitivity of detection, a substrate replacement step was incorporated into the protocol. The introduction of substrate replacement to the RT-QuIC protocol (**Figure 2**) increased the sensitivity (77%; 14 out of 18 samples in RT-QuIC were positive) and specificity (100%; none of the negative controls in RT-QuIC turned positive) of detection (see Table 1 from Cheng *et al.* [27]). Finally, all these steps (**Figure 3**) led to the optimization of a reliable and sensitive protocol for RT-QuIC detection of CWD prions, using specimen containing low levels of infectivity.

Substrate replacement was introduced after the first 25 h of RT-QuIC reaction, by replenishing reaction buffer containing fresh substrate and fresh fluorescence dye Th-T. By introducing the substrate replacement step in the protocol, the RT-QuIC reaction time was extended from 50 h to 75 h. As shown in **Figure 2** with the incorporation of substrate replacement, a significant improvement of prion amplification was observed.

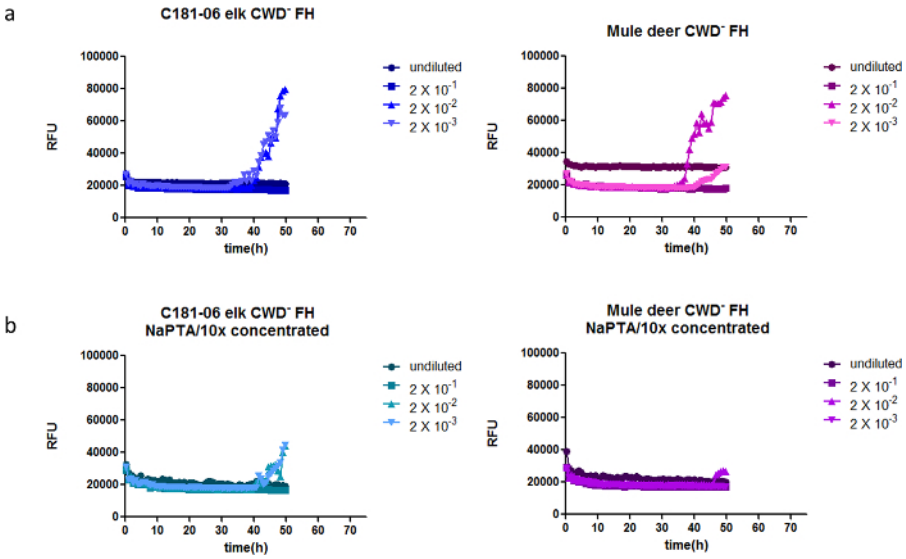

Figure 1: Reduced spontaneous conversion of mouse rPrP substrate in RT-QuIC seeded with purified CWD-negative fecal homogenate. Fecal homogenates of non-infected elk (C181-006) or mule deer were homogenized in feces extract buffer to obtain a final concentration of 10% (w/v). For purification, these fecal homogenates were processed and 10-times concentrated by NaPTA precipitation. The unpurified fecal homogenates (**a**), NaPTA-purified and concentrated forms (**b**) diluted as indicated were used to seed quadruplicate RT-QuIC reactions with mouse rPrP as a substrate. The y-axes show relative Th-T fluorescence units, the x-axes depict the reaction time. A reduction of spontaneous conversions was seen in NaPTA-purified fecal samples (**b**). Data used from Cheng *et al.* [27]. Please click here to view a larger version of this figure.

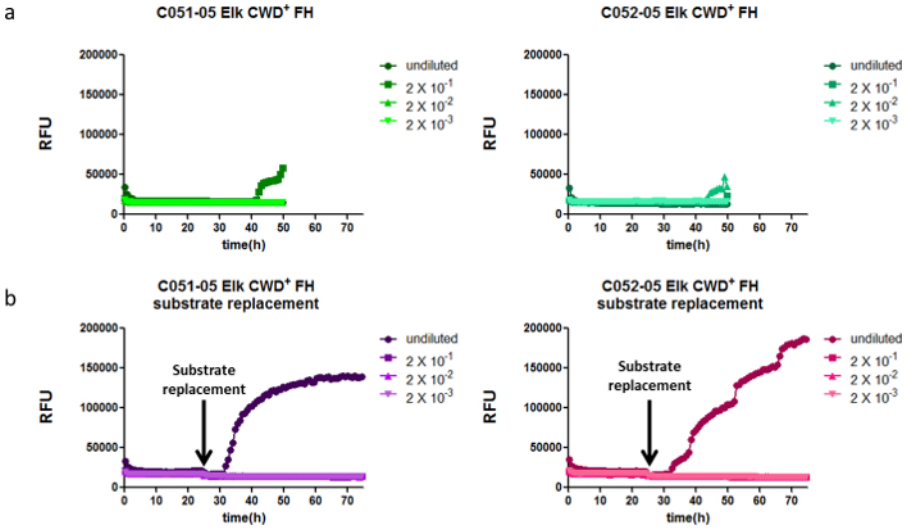

Figure 2. Improved detection of CWD prions in feces using substrate replacement. Fecal homogenates of individual elk (C051-05, C052-05) orally infected with CWD prions were purified and concentrated by NaPTA precipitation. NaPTA-treated samples were diluted between 2 x 10^{-1} to 2 x 10^{-3} used to seed quadruplicate RT-QuIC reactions with mouse rPrP substrate. The RT-QuIC assay was performed without substrate replacement (**a**) in a regular period of 50 h or with the incorporation of substrate replacement for an extended incubation period of 75 h (**b**). For the latter, substrate replacement was introduced after the first 25 h of RT-QuIC reaction. Ninety percent of the reaction volume was removed and replaced by freshly prepared RT-QuIC reaction mixture containing rPrP substrate and Th-T. By introducing the substrate replacement step in the protocol, the RT-QuIC reaction time was extended from 50 h to 75 h. Data used from Cheng *et al.* [27]. Please click here to view a larger version of this figure.

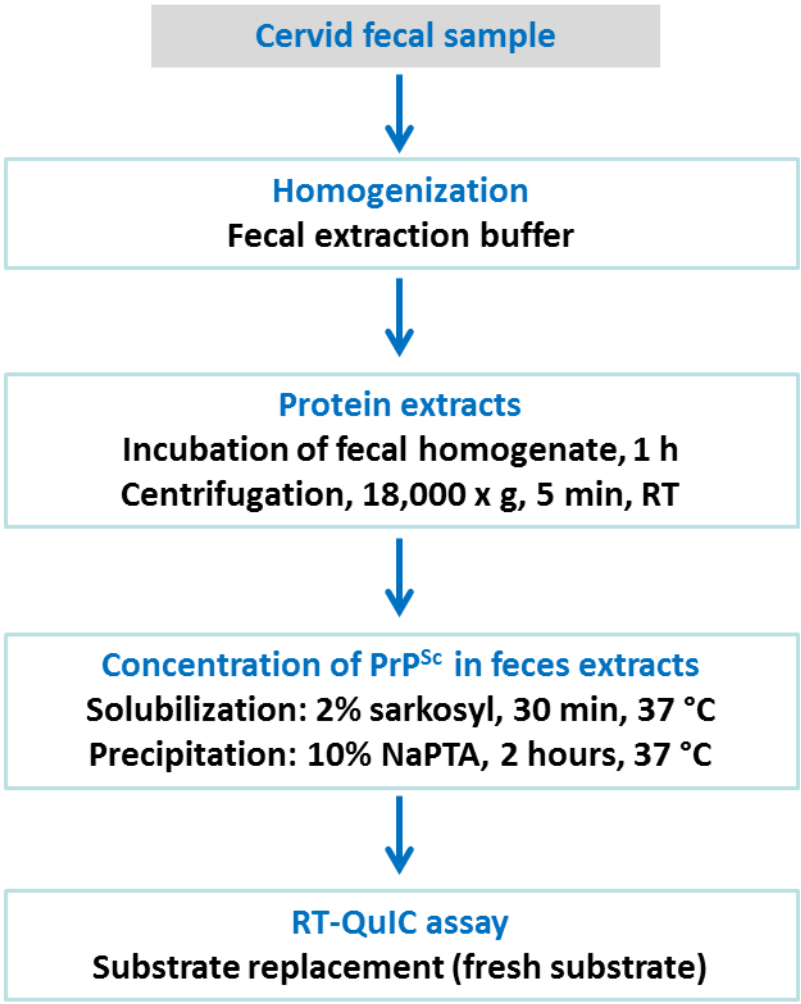

Figure 3: Flow diagram describing PrP^{Sc} fecal extraction from CWD-infected cervids and seeding activity and prion amplification using RT-QuIC assay. Fecal samples from CWD-infected animals are homogenized in a fecal extraction buffer, submitted to NaPTA precipitation method and tested by RT-QuIC assay. The latter was performed by introducing a substrate replacement step. The incorporation of NaPTA and substrate replacement steps resulted in a reduction of spontaneous conversion and strong improvement in seeding activity and prion amplification. Please click here to view a larger version of this figure.

Discussion

RT-QuIC was previously employed to detect CWD prions in urine and fecal extracts of orally infected white-tailed deer and mule deer [38]. The system shown in this manuscript is an adapted method of the RT-QuIC assay. Additional steps were incorporated into the "classical" RT-QuIC assay to improve the detection and sensitivity of the assay for CWD prions in fecal material of infected animals.

The low sensitivity of detection in feces extracts led us to the improvement of the RT-QuIC protocol. To achieve sensitive *in vitro* detection of prions in feces, critical steps were added for sample preparation in order to remove components which interfere with prion conversion and/or propagation and enhance the sensitivity of detection. Incorporating substrate replacement in the protocol of RT-QuIC and NaPTA/sarkosyl treatment was critical for detecting seeding activity in pre-clinical and clinical CWD-infected elk, and for enhancing the detection limits of prion conversion in feces extracts.

NaPTA precipitation is a common technique usually accompanied by sarkosyl extraction to isolate and concentrate PrP^{Sc} to a detectable level. NaPTA is known to preferably precipitate PrP^{Sc} over PrP^{C} [43] while sarkosyl is a detergent known to facilitate prion conversion at low concentrations in a cell free system [44]. In alignment with this, using a combination of NaPTA and sarkosyl might generate a higher yield of fibrillar aggregates *in vitro* as shown before [45,46,47]. This methodology has been incorporated previously into RT-QuIC assay to detect peripheral CWD prions successfully in specimen such as purified saliva [39] and whole blood [40]. Our study provides first evidence that incorporating NaPTA/sarkosyl purification in the protocol of fecal sample preparation enables CWD prion detection by RT-QuIC. Moreover, with this methodology we were able to reduce spontaneous conversion of rPrP substrate in CWD-negative fecal homogenates in RT-QuIC assay.

A potential mechanism has been proposed to explain the effect of substrate replacement [30]. In the first rounds of reaction (before the addition of fresh substrate), only a small amount of seeds is added to RT-QuIC reactions. While only a portion of rPrP is incorporated into the seeded reactions, the rest of the substrate could either be used up by interacting with the walls of reaction plate wells to form non-amyloid aggregates,

or be altered to a form which is less prone to be converted by seeded RT-QuIC products rather than the original seeds. As a result, the incorporation rate of rPrP to the seeds is slow, and the fibril formation is not detectable in the lag phase. As the seeded RT-QuIC products grown in the lag phase are finally elongated to reach a "fast assembly stage", prions can be amplified rapidly by segmentation through shaking or lateral addition of smaller aggregates. At this stage, the fresh substrate added to the reaction is readily incorporated into the seeded products rather than forming unspecific aggregates. The incorporation of the substrate replacement step in our protocol was a modification that increased sensitivity; it was useful to detect prion seeds in samples with a very low amount of PrP^{Sc} from pre-clinical animals and/or containing inhibitory compounds.

Using RT-QuIC assay rather than PMCA, which is the first *in vitro* protein misfolding amplification assay described [21], has several advantages. In PMCA the tubes are incubated and sonicated in a water bath contained within the sonicator; the tubes positioned at the periphery of the sonicator show less efficacy of amplification compared to the tubes positioned in the center [48]. The RT-QuIC system quaking seems to be easier to control. The use of a 96 well format is a real advantage of RT-QuIC, however, the mb-PMCA developed by Moudjo *et al.* [49,50], showed similar benefits but still is less used in PMCA.

As substrate, RT-QuIC uses recombinant rPrP for conversion; in contrast, PMCA in most cases uses normal brain homogenate. In addition, in RT-QuIC no sequence homology is required between seed and substrate [51-53].

The presence of cofactors is necessary for prion replication in PMCA and the resulting product is infectious. However, in RT-QuIC assay, the amplified PrP is not infectious. In *in vitro* amplification assays the occurrence of false positive reactions most probably because of spontaneous conversion of PrP^{C} is a recurring problem. Therefore, the assay and conditions used in this study were tailored to minimize such disturbances to maximize the difference between seeded rPrP-conversion and spontaneous conversion.

In conclusion, NaPTA/sarkosyl treatment enables removal of assay inhibitors in feces and reduces spontaneous conversion. Interestingly, the treatment did not hamper seeding activity of precipitatedPrP^{Sc}. In addition, the sensitivity of detection was significantly improved when substrate replacement was concomitantly adopted.

Disclosures

The authors have nothing to disclose.

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