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Aqueous extraction of formalin-fixed paraffin-embedded tissue and detection of prion disease using real-time quaking-induced conversion

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Abstract

Objective The goal of the research presented here is to determine if methods previously developed for the aqueous extraction of PrP^{Sc} from formalin-fixed paraffin-embedded tissue (FFPET) are applicable to the detection PrP^{Sc} by real-time quaking induced conversion (RT-QuIC). Previous work has utilized aqueous extraction of FFPET for detection of transmissible spongiform encephalopathies (TSEs) utilizing western blot and ELISA. This research extends the range of suitable methods for detection of TSEs in FFPET to RT-QuIC, which is arguably the most sensitive method to detect TSEs.

Results We found complete agreement between the TSE status and the results from RT-QuIC seeded with the aqueous extract of FFPET samples. The method affords the diagnostic assessment TSE status by RT-QuIC of FFPET without the use of organic solvents that would otherwise create a mixed chemical-biological waste for disposal.

Keywords Paraffin-embedded tissue, Prion, PrP, Spongiform encephalopathy, Transmissible spongiform encephalopathy, RT-QuIC

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases result from a conformational change in the host encoded prion protein, often termed PrP^{C} to denote the normal cellular conformation, into the disease associated formation (PrP^{Sc}) [1]. The diagnosis of a TSE is primarily conducted via immunodetection such as immunohistochemistry (IHC), western blot, and enzyme immunoassay. Formalin-fixed paraffin-embedded tissue (FFPET) is essential in the diagnosis of prion disease by IHC, and IHC is the standard by which all other diagnostic protocols for TSEs are judged. The advantages that IHC offers over other diagnostic approaches are numerous and include the ability to assess the amount and distribution of PrP^{Sc} within a sample as well as the preservation afforded by formalin fixation. However, IHC and the formalin-fixed tissues utilized for IHC do not typically couple well to amplification based approaches of prion disease detection. This limitation has the potential to hamper sensitivity of techniques compatible with FFPET as advances in amplification based techniques such as real-time quaking induced conversion (RT-QuIC) continue to enhance sensitivity. In this study, we apply an approach based on previous work utilizing FFPET and

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an aqueous extraction step for western blot and ELISA based detection of TSEs to the detection of TSEs using RT-QuIC [2–5].

Main text

Methods

The method for aqueous extraction described here is an extension of previously published methods for western blotting and ELISA based detection of PrPSc [3–5] and differs only in the method of detection that is detailed in the method section RT-QuIC. It should be noted that the samples were not selected, prepared, or run blinded.

Tissue samples

This study utilized archived FFPET samples from studies of scrapie in sheep, chronic wasting disease (CWD) in white-tailed deer (WTD) and transmissible mink encephalopathy (TME) in cattle as part of research conducted at the National Animal Disease Center-USDA-ARS (Ames, IA) [6, 7]. Animals were cared for and euthanized under National Animal Disease Center approved institutional animal care and use protocols #3432, 3347, or 3636.

Samples were collected in 10% neutral buffered formalin prior to standard processing into paraffin blocks, with time in formalin ranging from 7 days to ~ 450 days.

Sample preparation

Four 5 µm thick tissue section from each paraffin block were collected into a 1.5 ml centrifuge tube. To each, 150 µl of 50 mM Tris (pH 7.5), 1 mM EDT, and 0.5% Tween 20 was added. The tube was then placed at 100 °C for 10 min and immediately placed into a dry ice ethanol slurry until frozen. The 10 min boil/freeze cycle was repeated twice. The sample was then brought to 100 °C for an additional 10 min and immediately centrifuged at 3000×*g* for 10 min to separate the paraffin from the aqueous phase while also pelleting the tissue. If separation of paraffin was incomplete, the tube was placed at 100 °C for an additional 10 min and the centrifugation step repeated. The aqueous layer including the tissue pellet was then transferred to a clean 1.5 ml tube. At this point, sample volumes were ~ 120 µl. This sample was then sonicated for 10 intervals of 40 s with brief vortex mixing between steps to affect tissue disruption. The samples were then frozen at − 20 °C for later use.

Fresh-frozen tissue samples were prepared as previously described [8–11]. Briefly, brain homogenates (10%, w/v were prepared as whole homogenates of brainstem sections by bead beating (BB) homogenization with 1.0 mm silica beads in 1× Dulbecco's PBS pH 7.4. Samples were centrifuged briefly (1000*g* for 2 min) and supernatants stored at − 80 °C in aliquots.

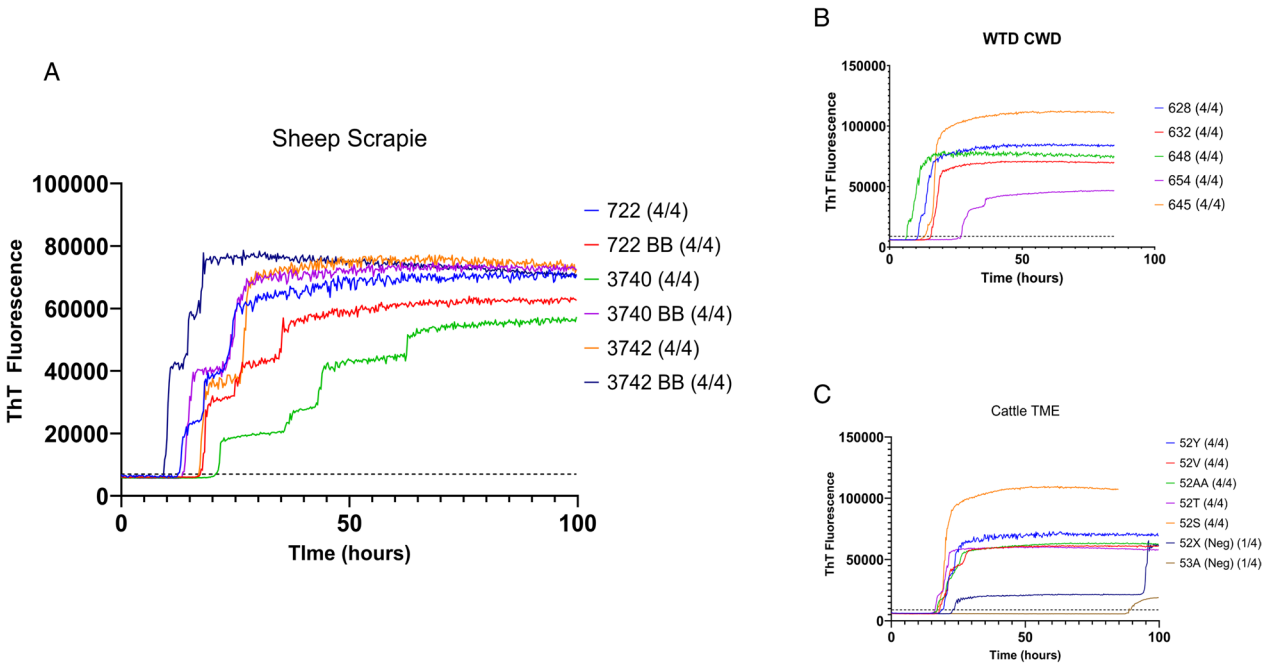

Fig. 1 RT-QuIC results for Sheep Scrapie (**A**), WTD CWD (**B**), and Cattle TME (**C**). **A** highlights the slightly reduced total fluorescence and increased lag time for FFPET samples relative to the fresh frozen samples that have been designated with "BB". The positive fluorescence cutoff is designated by a dashed line

RT-QuIC protocol

RT-QuIC reactions were performed as previously described [8–11]. The reaction mix was composed of 10 mM phosphate buffer (pH 7.4), 400 mM NaCl, 0.1 mg/ml recombinant BV prion proteins, 10 µM thioflavin T (ThT), and 1 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA). Aliquots of the reaction mix (98 µl) were loaded into each well of a black 96-well plate with a clear bottom (Nunc, Thermo Fisher Scientific) and seeded with 2 µl of the aqueous extraction of FFPET described above or in the case of fresh/frozen comparison samples a 10^{-2} dilution of 10% (w/v) BB brain homogenate. The plate was then sealed with plate sealer film and incubated at 42 °C in a plate reader (BMG FLUOstar Omega) with cycles of 1 min shaking (700 rpm double orbital) and 1 min rest for 100 h. ThT fluorescence measurements (excitation, 460 nm; emission 480 nm, bottom read, 20 flashes per well, manual gain 1400) were taken every 45 min. Each sample was analyzed as 2 repeats of 4 wells each for a total of 8 replicate RT-QuIC assays. To be determined to be positive, 2 wells of 4 must meet the criteria of positivity by Orru et al. [12–14].

Results

In this work we applied a method previously developed for western blot and ELISA detection of PrP^{Sc} in FFPET tissues to the preparation of samples for RT-QuIC. We found complete agreement between the known TSE status and the results from RT-QuIC detection of PrP^{Sc} in the aqueous extract of FFPET samples. These results are shown in Fig. 1 and Table 1. One negative control sample, #0009, tested positive in the RT-QuIC assay. This animal is from a scrapie free source and not sham inoculated. Based on this and the sensitivity of the RT-QuIC assay, we concluded that contamination may have occurred at the time of sections being cut. It was decided to recut those samples for 2 additional independent extractions. Both of those extracts tested as negative by RT-QuIC supporting the possibility that the initial positive result was due to contamination.

Table 1 Summary of FFPET RT-QuIC results compared to known TSE status

TSE (species)	Animal Number	TSE status	FFPET RT-QuIC
Scrapie (sheep)	721	+	+
	745	+	+
	744	+	+
	3740	+	+
	3742	+	+
Neg (sheep)	0009	–	–[a]
	0007	–	–
	3527	–	–
	0050	–	–
	0059	–	–
	0052	–	–
CWD (WTD)	628	+	+
	632	+	+
	648	+	+
	654	+	+
Neg (WTD)	107	–	–
	109	–	–
TME (cattle)	52S	+	+
	52V	+	+
	52Y	+	+
	52AA	+	+
	52T	+	+
Neg (cattle)	52X	–	–
	53U	–	–

[a] This sample initially tested positive. As indicated in the results additional sections were cut processed and analyzed, and in subsequent RT-QuIC was determined to be negative

Discussion

An alternative approach to preparing FFPET samples for RT-QuIC detection of prion disease does exist [15], and there are several reports of the use of FFPET coupled with RT-QuIC to detect Parkinson's disease [16–18], but these methods all differ by using xylene to deparaffinize the samples resulting in a mixed chemical-biological waste that must be disposed of properly. The approach presented here avoids this by utilizing an aqueous deparaffinization step.

It is worth noting that the unfixed tissue control samples all exhibited a shorter lag time and increased ThT fluorescence relative to that of the corresponding fixed tissues, both of which are indicative of the amount of PrP^{Sc} associated with the sample This may reflect an inhibitory effect of fixation on RT-QuIC, however, it is also possible that these differences arise as a result of the amount of tissue present in the FFPET extract being low compared to that present in a tissue sample prepared from fresh/frozen brain homogenate. There is no reasonable mechanism by which we can quantify the amount of tissue in a given paraffin embedded tissue and as such we cannot distinguish the origin of this empirical observation, but felt that it is worth noting for those interested in applying this approach.

In summary, this method for aqueous extraction of FFPET coupled to RT-QuIC affords the diagnostic assessment of formalin fixed samples by RT-QuIC without the use of organic solvents that would otherwise create a mixed chemical-biological waste for disposal.

Limitations

- Sample cross contamination could result in a false positive result. Specifically, the detection method, RT-QuIC, is sufficiently sensitive that the risk of sample contamination necessitates additional cleaning and use of clean forceps and new microtome blades for each sample as the sections are cut.
- The fluorescence of samples prepared from aqueous extraction of FFPET is lower than that of a fresh/frozen sample from the same animal indicating that the sensitivity may be reduced relative to the use of fresh/frozen samples.

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Author contributions
E.M.N. conceived the experimental approach and wrote the manuscript, S.H. conducted all RT-QuIC assays. J.J.G. provided tissue samples. All authors reviewed the manuscript. S.H. completed all work as a National Animal Disease Center, USDA-ARS employee. This work predates her employment at the Center for Veterinary Biologics, USDA-APHIS.

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Availability of data and materials
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC, USA) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL, USA) under the approval of the Institutional Animal Care and Use Committee of the National Animal Disease Center (protocol numbers 2415 and 2618).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interest.

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