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Autoreferát disertační práce

UNIVERZITA KARLOVA
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Intravitální diagnostika neurodegenerativních onemocnění

Intravital diagnostics of neurodegenerative diseases

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Abstract

Prionopathies, also called transmissible spongiform encephalopathies (TSE) and synucleinopathies are neurodegenerative diseases that are associated with the accumulation of misfolded proteins (prion and α-synuclein) mostly in the central nervous system. To this day, early and definite diagnosis remains unavailable during the patient's lifetime, mainly due to the absence of reliable biomarker which makes clinical diagnosis more challenging. Therefore, the gold standard in diagnostics remains direct *post-mortem* evaluation of misfolded proteins within brain tissue by western blot and immunohistochemistry. In the recent years, seeding amplification assays (SAAs) like Real-Time Quacking-Induced Conversion (RT-QuIC) emerged for ultra-sensitive *ante-mortem* diagnosis of neurodegenerative diseases. SAAs exploit ability of pathological misfolded proteins present in patient's samples to change the conformation and initiate aggregation of native recombinant protein substrate by prion-like seeding mechanism.

In the presented dissertation thesis, we exploited second-generation RT-QuIC assay (55°C, 700 rpm, cycles of 1 min double-orbital shaking and 1 min incubation) utilizing recombinant hamster shortened prion protein (rHAPrP90-231) to evaluate prion seeding activity in *post-mortem* TSE (n=38) and non-TSE (n=30) cerebrospinal fluid (CSF) and corresponding skin samples. In CSF, we were able to achieve 100% sensitivity and specificity after dilution of samples to remove the effect of present inhibitors. In the skin samples, the sensitivity and specificity of the assay was 89.5% and 100%, respectively. Interestingly, the analysis showed higher median prion seeding dose in the skin samples than in corresponding CSF. To further explore diagnostic potential of skin, we analyzed skin (head apex and ear lobe) from mice inoculated intracerebrally or subcutaneously with RML strain of prions. Subcutaneously infected mice showed positive RT-QuIC results in the skin from the apex and

ear lobe 12 days and 40 days before the onset of clinical symptoms, respectively. However, intracerebrally infected mice displayed positive prion seeding activity in the skin only after the onset of symptoms. Moreover, we examined prion seeding activity in two siblings with a genetic Creutzfeldt-Jakob disease with a novel five octapeptide repeats insertion (5-OPRI, R1-R2-R2-R3-R4-**R2-R2-R3-R3-R4**) in the *PRNP* gene. Duration of the disease was more than 10 years in both patients. In the first case, positive seeding activity was detected in every type of tested sample (frontal lobe, cerebellum, CSF, skin). On the contrary, in the second case, no RT-QuIC positivity was detectable suggesting the possible presence of different prion strain in patients with identical mutation. We successfully adopted a protocol for RT-QuIC analysis of formalin-fixed paraffin-embedded brain tissue of TSE patients and demonstrated its ability to detect prions in cohorts of TSE (n=30) and control non-TSE (n=30) patients.

Furthermore, we established RT-QuIC assay adapted for synucleinopathies (42°C, 400 rpm, cycles of 1 min double-orbital shaking and 1 min incubation). We validate α-syn seeding activity in 15 *post-mortem* brain homogenates and CSF samples with definite Dementia Lewy bodies (DLB, n=6), Alzheimer disease with Amygdala Lewy bodies (AD/ALB, n=3) and Creutzfeldt-Jakob disease with DLB (CJD/DLB) comorbidity (n=6) with 100% and 92.9% sensitivity, respectively. We also reported detection of α-syn higher seeding activity in a few non-TSE control samples. However, in two of them, secondary synucleinopathy was confirmed after the neuropathological reevaluation which was prompted by our RT-QuIC results.

Key words: diagnosis, neurodegenerative diseases, prion, CJD, α-synuclein, synucleinopathy, RT-QuIC

Abstrakt

Prionopatie, taktiež nazývané transmisívne spongiformné encefalopatie (TSE), a synukleínopatie predstavujú skupinu neurodegeneratívnych ochorení, ktoré sú asociované s akumuláciou nesprávne zložených proteínov (prión a α-synukleín) prevažne v centrálnom nervovom systéme. Kvôli absencii spoľahlivého biomarkera je včasná a definitívna diagnostika počas života pacienta nedostupná. Zlatým štandardom preto zostáva priama *post-mortem* detekcia patologicky zložených proteínov v mozgovom tkanive pomocou western blotu a imunohistochémie. Avšak v posledných rokoch sa pre ultra senzitívnu *ante-mortem* diagnostiku zaviedli metódy so spoločným názvom "seeding amplification assays (SSAs)" akou je aj „Real-Time Quacking-Induced Conversion" (RT-QuIC). SAAs využívajú schopnosť patologicky zložených proteínov, ktoré sú prítomné v pacientskej vzorke, zmeniť konformáciu a iniciovať agregáciu monomérneho rekombinantného proteínu v substráte vďaka "prion-like" mechanizmu.

V prezentovanej dizertačnej práci sme analyzovali prión konvertujúcu aktivitu v *post-mortem* TSE (n=38) a non-TSE (n=30) vzorkách cerebrospinálneho moku (CSF) a korešpondujúcich vzoriek kože pomocou druhej generácie RT-QuIC metódy (55°C, 700 rpm, cykly 1 min dvoj orbitálneho trepania a 1 min inkubácia) s využitím rekombinantného skráteného priónového proteínu škrečka zlatého (rHAPrP90-231) ako substrátu. Vo vzorkách CSF, sme boli schopní dosiahnuť 100 % senzitivitu a špecificitu až po nariedení vzoriek, ktoré odstráni efekt inhibítorov prítomných vo vzorke. Vo vzorkách kože sme dosiahli 89,5 % senzitivitu a 100% špecificitu. Prekvapivo, analýza vzoriek ukázala vyššiu priemernú prión konvertujúcu aktivitu vo vzorkách koží než v CSF. Na bližšie preskúmanie diagnostického potenciálu kože, sme analyzovali vzorky kože (z apexu hlavy a ušného laloku) z myší, ktoré boli intracerebrálne alebo subkutánne inokulované priónovým kmeňom RML. Subkutánne inokulované myši

vykazovali RT-QuIC pozitívny signál v koži z apexu a ušného laloku 12 a 40 dní pred nástupom klinických príznakov. Avšak myši, ktoré boli inokulované intracerebrálne vykazovali pozitívnu prión konvertujúcu aktivitu v koži až po nástupe symptómov. Navyše sme otestovali prión konvertujúcu aktivitu u dvoch súrodencov s genetickou Creutzfeldt-Jakobovou chorobou (CJD) s novou mutáciou piatich oktapeptidových repetitívnych inzercií (5-OPRI, R1-R2-R2-R3-R4-**R2-R2-R3-R3-R4**) v *PRNP* géne. U obidvoch pacientov bola doba ochorenia dlhšia ako 10 rokov. V prvom prípade sme detegovali prión konvertujúcu aktivitu v každej testovanej vzorke (frontálny lalok, mozoček, CSF a koža). Naopak, v druhom prípade sme nedetegovali žiadnu RT-QuIC pozitivitu čo naznačuje možnú prítomnosť iného priónového kmeňa u pacientov s identickou mutáciou. Úspešne sme zaviedli protokol na RT-QuIC analýzu parafínových vzoriek mozgu fixovaných vo formalíne od pacientov s TSE a demonštrovali schopnosť metódy detegovať prióny v kohorte pacientov s TSE (n=30) a kontrolných non-TSE (n=30) pacientov.

Okrem toho sme zaviedli RT-QuIC metódu adaptovanú na synukleínopatie (42°C, 400 rpm, cykly 1 min dvoj orbitálneho trepania a 1 min inkubácie). Validovali sme α-syn konvertujúcu aktivitu u 15 *post-mortem* vzoriek mozgových homogenátov a CSF s definitívnou diagnózou Demencie s Lewyho telieskami (DLB, n=6), Alzheimerovej choroby s amygdala Lewyho telieskami (ALB, n=3) a Creutzfeldt-Jakobovej choroby s DLB (CJD/DLB, n=6) komorbiditou so 100 % senzitivitou a 92,9 % špecificitou. Taktiež sme reportovali detekciu vyššej α-syn konvertujúcej aktivity u pár non-TSE kontrolných vzoriek. Avšak pri dvoch kontrolných vzorkách bola potvrdená sekundárna synukleínopatia po neuropatologickej reanalýze na podnet našich RT-QuIC výsledkov.

Kľúčové slová: diagnostika, neurodegeneratívne ochorenia, prión, CJCH, α-synukleín, synukleínopatia, RT-QuIC

1 Introduction

Early and reliable *ante-mortem* diagnosis of neurodegenerative diseases is crucial not only for patients and their families in order to not misdiagnose treatable disease, but also for the management of prevention of secondary transmission, especially in prion diseases. However, *ante-mortem* diagnosis often relies on the evaluation of clinical symptoms that can be supported by familial history, magnetic resonance imaging (MRI), electroencephalography (EEG) or on the levels of proteins such as 14-3-3, *p*-tau and *t*-tau in cerebrospinal fluid (CSF) (Chatzikonstantinou *et al*., 2021). However, the sensitivity and specificity of these methods is low as symptom are overlapping with other neurodegenerations and the protein content in *ante-mortem* CSF is lower (Chohan *et al*., 2010; Panigaj *et al*., 2011). Therefore, the gold standard for definitive diagnosis remains direct detection of pathologically misfolded proteins like prion protein (PrP^{TSE}) and alpha-synuclein ($\alpha\text{-syn}^{D}$) by immunohistochemistry during the autopsy. Diagnosis of prion diseases is complemented also by Western blot (WB) analysis.

In the last decade, new ultra-sensitive methods which exploit the seeding 'prion-like' ability of misfolded proteins emerged as a diagnostic tool to improve *ante-mortem* diagnosis. Real-Time Quacking-Induced Conversion (RT-QuIC) assay developed by the laboratory of Dr. Byron Caughey (Atarashi *et al*., 2007) utilize recombinant prion protein (rPrP) as a source of monomeric PrP for the template driven conversion. Reaction mix, containing rPrP and fluorescent dye Thioflavin T (ThT), is usually seeded with patient's sample such as brain homogenate (BH), CSF, skin homogenate or olfactory mucosa swab in quadruplicates using 96-well plate (Fig. 1). The samples undergo cycles of intermitting shaking which breaks newly formed fibrils and incubation period during which amyloid aggregated can form. Increase of ThT fluorescence due to its binding to the aggregates is monitored in real time.

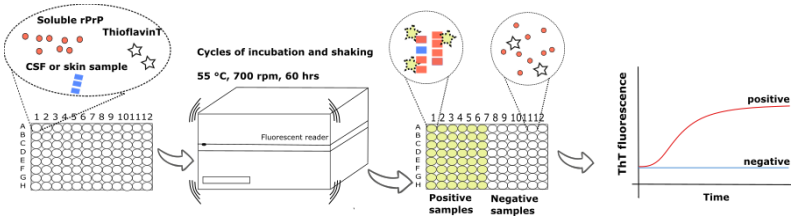

Figure 1. Schematic overview of Real-Time Quacking-Induced Conversion assay (RT-QuIC).

Improved RT-QuIC assay for prion diseases was shown to provide 82 – 97% sensitivity and 99 – 100% specificity in diagnosis of prionopathies *intra-vitam*. The RT-QuIC assay was recently adapted for other neurodegenerations including synucleinopathies utilizing recombinant alpha-synuclein wild-type (rα-Syn (WT)) as a substrate. In contrast, with prion RT-QuIC, the α-syn conversion is promoted by addition of mixing beads into the reaction. Fairfoul *et al*. (2016) utilized 'prion-like' seeding activity to detect α-syn^{D} in brain and CSF with 95% sensitivity and 92 – 100% specificity. However, despite the number of studies that reported high sensitivity and specificity of the assay, RT-QuIC assay for synucleinopathies remains much less developed and more studies are needed to understand its real diagnostic potential.

2 Aims

Early and sensitive detection of misfolded proteins is crucial for the prognosis and the treatment management of neurodegenerative diseases. However, *intra-vitam* diagnosis is still challenging as many neurodegenerations overlap symptoms, particularly at clinical onset, leading to frequent misdiagnosis. For that reason, direct neuropathological examination of amyloid aggregates in brain remains the gold standard for definite diagnosis.

Therefore, our main aim was to validate the diagnostics potential of RT-QuIC assay analyzing different patient's samples with definite diagnosis of prionopathy or synucleinopathy in our laboratory.

Our partial aims were:

1. Purification of recombinant rHAPrP90-231 and rBVPrP (full-length) for prion RT-QuIC and rα-Syn (WT) for α-syn RT-QuIC assay.
2. Validation of prion RT-QuIC utilizing corresponding *post-mortem* cerebrospinal fluid (CSF), skin, and archive formalin-fixed paraffin-embedded brain samples.
3. Assessment of the RT-QuIC sensitivity with skin samples and usefulness of the assay during the asymptomatic stage utilizing mouse model.
4. Validation of prion RT-QuIC in patients with a rare genetic form of prion disease.
5. Validation of α-syn RT-QuIC in *post-mortem* brain and CSF samples.

3 Methods

Purification of rPrP: Recombinant short Syrian hamster prion protein (rHAPrP90-231) and full-length Bank vole prion protein (rBVPrP) were expressed in *E. coli* (Rosseta^{TM} (DE3)) using overnight Autoinduction System (Novagen). rPrP was isolated from inclusion bodies by lysis of bacterial cells using BugBuster master mix. Protein was purified by immobilized metal affinity chromatography using Ni-NTA Fast Flow resin beads (Cytiva). Protein was washed with 6 M guanidine hydrochloride (GndHCl) and refolded by slowly replacing denaturation conditions with physiological. The protein was eluted in linear gradient of 0 – 500 mM imidazole. The concentration was determined by dividing absorbance of the protein at 280 nm with theoretical extinction coefficient of 1.4 $mg/ml^{-1}cm^{-1}$ (rHAPrP90-231) or 2.7 $mg/ml^{-1}cm^{-1}$ (rBVPrP).

Purification of r-αSyn (WT): rα-Syn (WT) with 6x His-Tag on N-terminal was expressed in *E. coli* BL21 (DE3) using overnight Autoinduction system (Novagen). Protein was isolated from periplasm by osmotic shock. After resuspension in 40% saccharose, bacterial cells were lysed with ice-cold mQH_2O. Protein was purified using nickel affinity chromatography on 5 ml HisTrap^{TM} FastFlow (Cytiva) column. The protein was eluted by linear gradient of 50 – 500 mM imidazole. In the second step, the protein was purified and concentrated on anion exchange column (Cytiva). The concentration was determined using extinction coefficient of 0.36 $mg/ml^{-1}cm^{-1}$.

Mice experiment: 80 mice were inoculated either intracerebrally (i.c.) with 0.1% BH with RML prions or subcutaneously (s.c.) with 0.2% BH with RML prions. Controls were inoculated with PBS, pH 7.4. Three inoculated mice and two controls were housed together. Five intracerebrally inoculated control mice were placed in one additional cage. Mice were sacrificed every 28 or 42 days, depending on the inoculation route. BH, skin from the head apex and from ear were analyzed by RT-QuIC assay.

Preparation of samples for RT-QuIC: For prion RT-QuIC, 10% brain homogenates (BH) were diluted in PBS, 1x N-2 supplement (Gibco)

and 0.1% SDS and analyzed in end-point dilution $5x10^{-6}$ – $5x10^{-12}$. For α-syn RT-QuIC, BH were diluted either in PBS or in a buffer containing PBS, 1x N-2 supplement and 0.025% SDS. Samples were analyzed in 10^{-2} – 10^{-8} dilution. CSF samples were analyzed undiluted and 10x diluted in PBS for both assays. Skin was prepared as previously described (Orrú *et al*., 2017). Pieces about 30 – 60 mg were lysed by 0.25% collagenase A (Roche) over 4 hrs at 37°C and homogenized with sonication. 10% skin homogenate was diluted 10x or 100x (mice). Formalin-fixed paraffin-embedded (FFPE) brain samples were treated with xylene and rehydrated with graded ethanol washes (100%, 95% and 70%) as described (Hoover *et al*., 2016). FFPE tissue was analyzed in 10^{-2} – 10^{-8} dilution.

Prion RT-QuIC: Prion seeding activity was analyzed using second-generation RT-QuIC assay. Reaction mix was composed of 119 mM phosphate buffer (PB), 1 mM EDTA, 130 mM NaCl, 10 µM ThT and 0.1 mg/ml of rPrP. For CSF analysis, it was also supplemented with 0.002% SDS. 98 or 85 µl of mix was seeded with 2 µl of brain/skin or 15 µl of CSF in quadruplicates. Samples were subjected to cycles of double-orbital shaking (1 min, 700 rpm) and incubation (1 min) at 42°C/55°C over 48 or 60 hrs. The fluorescence was read every 15 min.

Alpha-synuclein RT-QuIC: Samples were analyzed using protocol by Groveman *et al*. (2018). Reaction mix was composed of 40 mM PB, pH 8.0, 170 mM NaCl, 10 µM ThT, 0.1 mg/ml rα-Syn (WT). For CSF, 0.0015% of SDS was added to the mix. The reaction was supplemented with 6 silica beads (OPS Diagnostics). The reaction was seeded in the same manner as for prion. The plate underwent cycles of double-orbital shaking (1 min, 400 rpm) and incubation (1 min) at 42 °C over 48 or 60 hrs.

Analysis of data: To analyze data from RT-QuIC assay, we evaluated four parameters – max ThT fluorescence, time to threshold (TTT), lag time, and area under the curve (AUC). Max ThT fluorescence was determined from the mean of four wells of tested sample.

4 Results

Detection of prion seeding activity in post-mortem ventricular CSF: When tested undiluted, 7 post-mortem TSE CSF samples (VV1, VV2, MV1, VPSPr, GSS and two MM1) gave negative RT-QuIC result. However, after dilution all TSE samples (n=38) displayed positive ThT signal suggesting 100% sensitivity. Control, non-TSE samples (n=30) gave ThT signal below calculated threshold corresponding to 100% specificity. Although, after dilution, few control samples showed elevated fluorescent signal in one well which resulted in higher threshold. The RT-QuIC analysis was repeated for those samples. After, ThT signal dropped (Fig. 2).

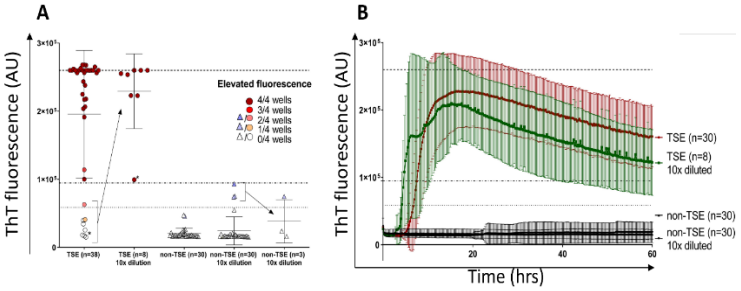

Figure 2. RT-QuIC analysis of post-mortem CSF samples. (A) Max ThT fluorescence of TSE and non-TSE samples. (B) The mean ThT fluorescence signal of undiluted TSE (red), diluted TSE (green) and control (black and grey) CSF samples.

Detection of prion seeding activity in *post-mortem* skin samples utilizing protocol for brain homogenates: Out of 38 skin samples, 31 were classified as positive suggesting 81.6% assay sensitivity. Seven TSE skin samples, specifically with MM1 (n=4), MV1 (n=1), VV1 (n=1) phenotype and gCJD with GSS (n=1), provided signal in two or more wells, but did not reach the threshold and were classified as negative. Analysis of non-TSE skin (n=30) samples gave negative signal below the established threshold suggesting the 100% assay specificity. However, five control samples, specifically FTLD-UPS, DLB, syn., AD and H/ABI cases, gave higher ThT signal above the SD (Fig. 3).

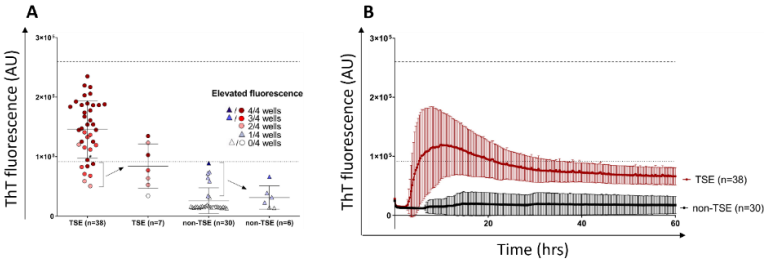

Figure 3. RT-QuIC analysis of post-mortem skin samples. (A) Max ThT fluorescence of TSE and non-TSE samples. (B) The mean ThT fluorescence signal of the samples.

Determination of RT-QuIC ability to detect prions before the occurrence of the disease symptoms in animal model: RT-QuIC analysis of mice samples showed differences in spread of PrP^{TSE} and substantially also differences in RT-QuIC prion seeding activity. The onset of the clinical symptoms in i.c. inoculated mice was faster and the RT-QuIC positivity in skin from apex and ear was detected only after the onset of symptoms (Fig. 4). In s.c. inoculated mice, the clinical onset of symptoms was slower, but the RT-QuIC positivity in skin apex and ear was detectable shortly before the onset of symptoms (Fig. 5). However, in the brain the prion seeding activity was detectable 84 days for i.c. and 68 days for s.c. inoculated mice before the onset of symptoms.

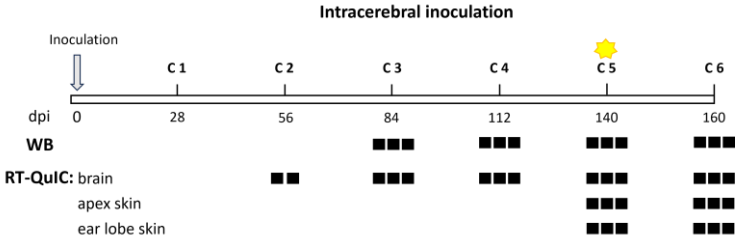

Figure 4. Schematic diagram of time points when i.c. inoculated mice were sacrificed. Proteinase K resistant PrP^{TSE} in brain was detectable 84 dpi by WB. The positive seeding activity was detected 56 dpi in brain, and 140 dpi in skin

from apex and ear. Black squares – number of mice with positive signal. The onset of clinical symptoms is marked in yellow asterisk. dpi – days post inoculation

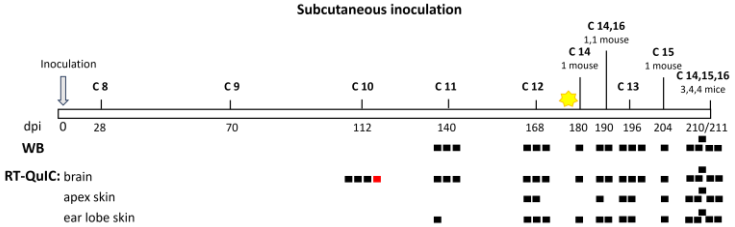

Figure 5. Schematic representation of s.c. inoculated mice and the time point they were sacrificed. The proteinase K resistant PrP^{TSE} was detected 140 dpi by WB. The positive prion seeding activity was detected 112 dpi in brain, 168 dpi in skin from apex and 140 dpi in skin from ear. One mouse gave false-positive RT-QuIC result 112 dpi (red square). The onset of clinical symptoms is marked with asterisk. Black squares – number of mice with positive signal. dpi- days post inoculation.

Detection of prion seeding activity in archive formalin-fixed paraffin embedded brain tissue: Prion seeding activity was detected in all FFPE samples from TSE patients (n=30) up to 10^{-3} dilution, however after that, the seeding activity noticeably decreased. At 10^{-8} dilution, none of the samples exhibited the specific aggregation. The lowest overlap between the TSE and control groups was seen at 10^{-4} dilution which was chosen for the of RT-QuIC outcome analysis. Two TSE (sCJD VV2 and GSS) cases gave ThT fluorescence signal below the threshold while conversely four non-TSE (FTLD-UPS, FTLD-tau, AD and ND-A) cases gave higher ThT fluorescence signal above the SD of the control group and were reanalyzed (Fig. 6).

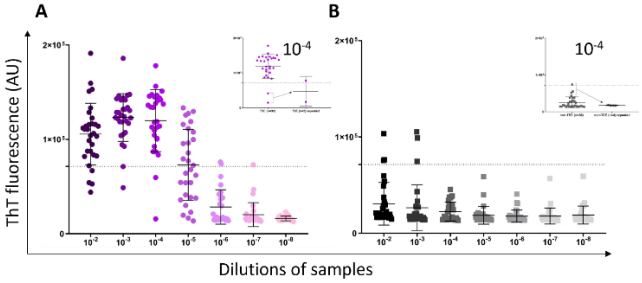

Figure 6. Dot plot of max ThT fluorescence intensity for TSE and non-TSE FFPE samples. Two TSE and four non-TSE samples were reanalyzed (inserted graphs) by RT-QuIC and the mean fluorescence from four wells was plotted in the graph. Dotted line represents the calculated positivity threshold.

Retrospective RT-QuIC analysis of samples from patients with rare genetic Creutzfeldt-Jakob disease: *Post-mortem* samples from two siblings with gCJD and a specific genetic mutation of five octapeptide repeats insertions (5-OPRI, R1-R2-R2-R3-R4-**R2-R2-R3-R3-R4**) in the *PRNP* gene encoding the prion protein were examined for prion seeding activity by RT-QuIC. The analysis was carried out on BH samples from the frontal lobe (Fig. 7), ventricular CSF, skin samples, and FFPE cerebellum tissues. Interestingly, in the case 1 all patient samples showed positive RT-QuIC seeding activity. On the other hand, the samples from case 2 did not show any positive specific aggregation.

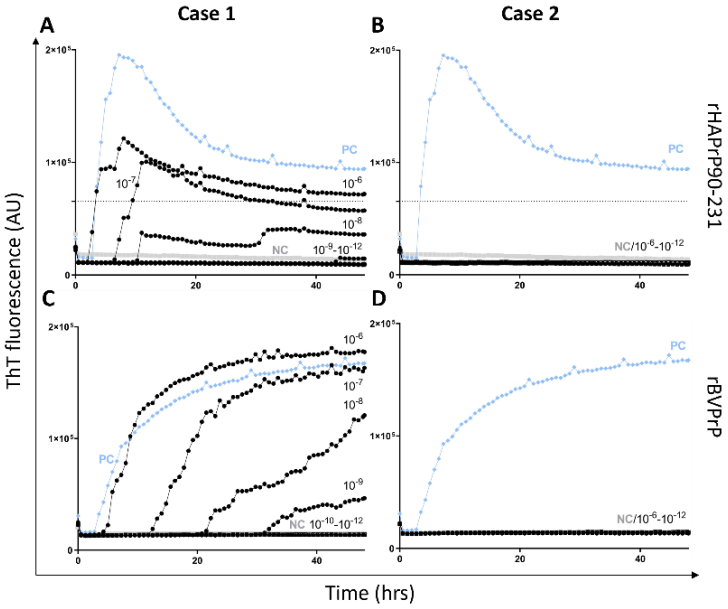

Figure 7. Representative RT-QuIC analysis of BH dilutions from two siblings with 5-OPRI gCJD. The prion seeding activity was tested using rHAPrP90-231. To confirm the results analysis was repeated with rBVPrP which is supposed to be more sensitive for genetic prionopathies. Samples from the case 2 did not showed positive RT-QuIC result using both substrates. Positive control (PC) from sCJD MM1 brain was used to compare classical CJD case with a rare genetic form.

Detection of α-syn^{D} seeding activity in brain homogenate samples using RT-QuIC: At first, *post-mortem* BH (n=15) were analyzed diluted in PBS, pH 7.4. However, this approach led to lower ThT signal, while synucleinopathy (DLB, AD/ALB, CJD/DLB) samples (n=15) were overlapping with control samples from patients with other neurodegenerations (n=17) and from corneal donors (n=17) (Fig. 8A). Therefore, we have modified the protocol to enhance the ThT signal with low concentration of SDS (0.0005%). This approach led to 100% sensitivity with synucleinopathy samples hitting the detection limit of the reader. Five control samples showed higher ThT signal above the

calculated threshold suggesting 85.3% specificity. However, two of positive AD samples were after immunohistological reevaluation reclassified as comorbidity synucleinopathies improving the assay specificity to 91.2% (Fig 8B).

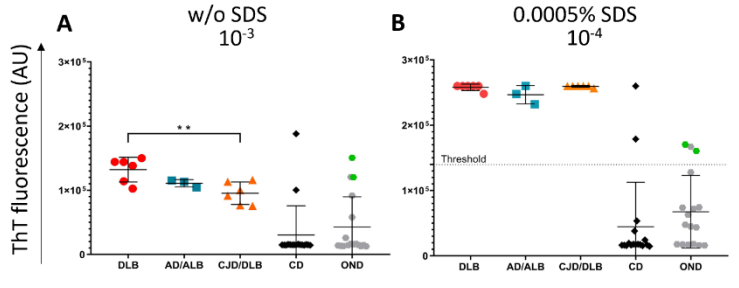

Figure 8. Dot plot analysis of max ThT fluorescence intensity detected in BH using two different RT-QuIC protocols. Samples were analyzed in 10^{-3} and 10^{-4} dilution. Green circles – samples reclassified as synucleinopathies.

Detection of α-syn^{D} seeding activity in the ventricular cerebrospinal fluid using RT-QuIC: To validate the seeding activity in CSF, 14 *post-mortem* corresponding samples obtained at autopsy were analyzed according to previously published protocol (Groveman *et a*l., 2018). When tested undiluted, one CJD/DLB comorbidity sample did not reach the calculated threshold and was classified as negative, indicating 92.9% assay sensitivity. The specificity corresponded to 83.3% with three samples, specifically two AD cases and one sample not defined, gave ThT signal above the threshold (Fig. 9A). To decrease the inhibitors of the assay, that are often present in *post-mortem* CSF, the samples were analyzed also diluted 10x in PBS, pH 7.4. However, after the dilution the sensitivity of the assay lowered. One AD/ALB and one CJD/DLB comorbidity did not reach the calculated threshold, corresponding to 85.7% assay sensitivity. But the specificity of the assay was higher for diluted CSF samples corresponding to 94.4%. Only one control AD sample gave fluorescence signal above the threshold (Fig. 9B).

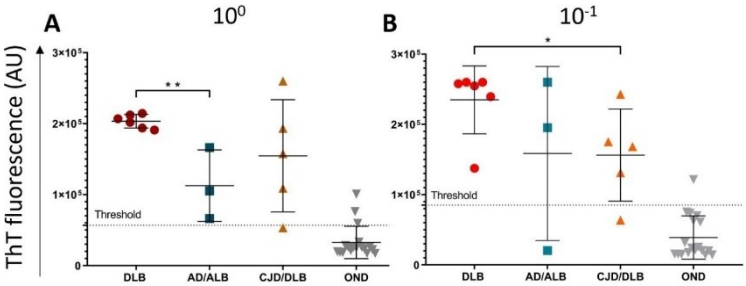

Figure 9. Dot plot of max ThT fluorescence intensity detected in undiluted and diluted CSF samples by RT-QuIC.

5 Discussion

To shed a light on the complexity of the diagnosis of neurodegenerations, we have analyzed different types of samples using different approaches to exploit the potential of RT-QuIC assay as a diagnostic tool for prionopathies and synucleinopathies. In retrospective study, we have analyzed prion seeding activity in *post-mortem* CSF samples and corresponding skin. The analysis was complicated by the fact that *post-mortem* CSF differs from the *ante-mortem* CSF. The concentration of total protein can be 20x higher after the death (Mangin *et al*., 1983). Therefore, we have analyzed CSF samples both undiluted and diluted to decrease the concentration of inhibitors (e.g. brain polar lipids, blood, plasma) that can be present *in post-mortem* ventricular CSF (Orrú *et al*., 2012; Hoover *et al*., 2017) and to avoid false-negative results. Interestingly, control samples that were 10x diluted gave higher ThT background and three control samples showed notably elevated ThT fluorescence in one or two wells. However, after repeating analysis, the max ThT signal of these samples lowered. The RT-QuIC analysis of *post-mortem* skin samples was more complex due to higher natural heterogeneity. The skin was obtained at autopsy from the area behind the ear, before opening the skull to avoid cross-contamination. This area produced the best results in different studies (Xiao *et al.,* 2021). At the beginning, we have tried various protocols to obtain the best results. Utilization of just 2 µl of sample to seed the reaction and inclusion of N-2 supplement led to the marked inhibition of spontaneous aggregation of control group samples while preserving good aggregation response in positive samples. Recently, similar sensitivity and specificity, as we were able to achieve utilizing skin samples, were reported also in the large-scale study by Zhang *et al*. (2024). To further clarify the RT-QuIC diagnostical potential we utilized mouse model. RT-QuIC assay was performed utilizing brain and skin tissue (head apex and ear lobe) at different time points after the mice were inoculated either intracerebrally or subcutaneously with RML prions or PBS as controls. Our results indicate differences in the spread of PrP^{TSE} and prion converting activity in intracerebrally and subcutaneously inoculated

mice. The course of the disease in intracerebrally inoculated mice was faster and the RT-QuIC positive prion seeding activity of the skin (apex and ear lobe) samples was detected only after the onset of clinical symptoms. On the contrary, in mice inoculated subcutaneously, the spread of prions was slower, but the positive RT-QuIC signal was detectable shortly before the occurrence of the symptoms. To further explore the application of RT-QuIC, we have established protocol for the detection of prion seeding activity in archive FFPE brain samples. We successfully detected prion seeding activity in all tested samples. This approach allows us to investigate seeding activity in different areas of the brain, that are usually not available as frozen BH. To our best knowledge, only Hoover *et al*. (2016) previously reported detection of prion seeding activity in FFPE obex samples from deer infected with chronic wasting disease by RT-QuIC. The RT-QuIC sensitivity varies between laboratories (Shi *et al*., 2021) when analyzing genetic prionopathies. Therefore, we analyzed the prion seeding activity in frontal lobe, cerebellum, ventricular CSF, and skin samples from two siblings (case 1 and 2) diagnosed with a novel 5-OPRI mutation genetic CJD (Schmitz *et al*., 2017). We have reported ambivalent results, when the case 1 displayed positive RT-QuIC seeding activity in all tested samples. On the other hand, samples from the case 2 did not show any positive RT-QuIC seeding activity. Similar observations were reported also by others (Mok *et al*., 2021; Hamada *et al*., 2023). These results indicate great heterogeneity not only between the clinical onset and symptoms but perhaps also in prion characteristics present in individual gCJD OPRI patients. Our last aim was to establish RT-QuIC assay for synucleinopathies in our laboratory. To examine complexities of co-pathologies we have analyzed archived *post-mortem* BH and CSF samples of patients with primary or secondary, concomitant, synucleinopathy. We confirmed 'prion-like' seeding activity in frontal lobe in all tested samples, especially in patients with a rare Alzheimer disease with amygdala Lewy bodies. Moreover, to our best knowledge, we are the first to analyze both α-syn and prion seeding activity in BH with CJD/DLB co-pathology utilizing specific RT-QuIC assays. We also reported positive ThT signal in three AD cases which was also observed by others (Manne *et al*., 2019; Bentivenga *et al*., 2024). To rule out the

possibility that the seeding activity in these cases was cause by α-syn^{D} aggregates, the brain FFPE samples we neuropathologically reanalyzed by immunohistochemistry and other areas, than frontal lobe, were examined. Indeed, in two patients secondary synucleinopathy was confirmed, improving the specificity of the assay. Therefore, we suggest paying special attention to cases with Alzheimer disease where α-syn^{D} can be present due to comorbidity or the aggregation of synuclein substrate may be occasionally initiated by cross seeding on the present amyloid fibrils. The RT-QuIC analysis of the corresponding *post-mortem* ventricular CSF samples was more straightforward and the differences between synucleinopathy groups were more significant comparing to BH samples. We have been able to confirm seeding activity in all examined samples including secondary synucleinopathies (AD/ALB and CJD/DLB). Also, Hall *et a*l. (2022) reported less robust RT-QuIC read outs when testing samples from patients with amygdala or brainstem restricted LB.

6 Conclusion

As of today, there is still lack of early and reliable diagnosis of neurodegenerative diseases during the patient's lifetime and the definite diagnosis is made only *post-mortem* during the autopsy. Therefore, our main aim of presented dissertation thesis was to examine the prion seeding activity in different types of patient's tissues (brain, CSF, and skin) utilizing ultra-sensitive Real-Time Quacking-Induced Conversion assay, to assess its diagnostic potential. Moreover, we established RT-QuIC assay adapted to α-syn^{D} in our laboratory to further investigate the utilization of its 'prion-like' seeding activity for laboratory diagnostics of synucleinopathies. We believe that our efforts contribute to better understanding of diagnostic potential of seed amplification assays which will help to introduce these methods into clinical practice as diagnostic tools for early diagnosis of neurodegenerative diseases.

- We have successfully purified rHAPrP90-231 and rBVPrP (full-length) for prion RT-QuIC assay along with rα-Syn (WT) for the assay adapted to synucleinopathies. Subsequently, we tested the suitability of the purified recombinant protein substrates through quality control assay utilizing RT-QuIC.
- We have confirmed the presence of prion seeding activity in *post-mortem* CSF and corresponding skin samples in the retrospective study. Moreover, we have determined the median seeding dose of CSF and skin samples across various CJD phenotypes.
- We have assessed the diagnostic potential of skin samples in the presymptomatic stage of the disease by analyzing samples (brain, skin from apex and ear lobe) from mice sacrificed at different time points after inoculation. We also compared the differences caused by different inoculation routes.

- We have established RT-QuIC assay employing formalin-fixed paraffin-embedded (FFPE) archive brain tissue.
- We have evaluated prion seeding activity in two siblings with genetic CJD with a novel five octapeptide repeat insertions (5-OPRI) in the *PRNP* gene. We have examined different tissue types (frontal lobe, ventricular CSF, ski and FFPE cerebellum tissue) utilizing different RT-QuIC substrates. We were able to detect positive seeding activity only in one sibling suggesting possible presence of two different prion strains.
- We have established and optimized RT-QuIC assay adapted to detect α-syn^{D} seeding activity in patient's samples.
- We have analyzed α-syn^{D} seeding activity in *post-mortem* BH and CSF from patients with definite primary or secondary synucleinopathy. Moreover, we have detected α-syn^{D} co-pathology in two control patients with definite diagnosis of Alzheimer disease.
- We have confirmed high-diagnostic potential of various tissue types and possible usefulness of RT-QuIC assay in *ante-mortem* diagnosis of prionopathies and synucleinopathies.

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List of publications

1. Publications *in extenso* which are the basis for dissertation thesis

Detection of prions in matching *post-mortem* skin and cerebrospinal fluid samples using second-generation real-time quaking-induced conversion assay.

Baranová S., Moško T., Brůžová M., Haldiman T., Kim Ch., Safar J.G., Matěj R., Holada K.

Scientific reports. 2024, doi.org/10.1038/s41598-024-56789-6

Impact factor: 4.6

Detection of Prions in Brain Homogenates and CSF Samples Using a Second Generation RT-QuIC Assay: A Useful Tool for Retrospective Analysis of Archived Samples.

Moško T., Galušková S., Matěj R., Brůžová M., Holada K.

Pathogens, 2021, **doi.org/10.3390/pathogens10060750**

Impact factor: 3.7

New possibilities of laboratory diagnostics of diseases associated with amyloid formation.

Galušková S., Moško T., Dušek P., Matěj R., Holada K.

Česká a Slovenská Neurologie a Neurochirurgie, 2021, doi.org/10.48095/cccsnn2021334

Impact factor: 0.5