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Accurate detection of pathologic α-synuclein in CSF, skin, olfactory mucosa, and urine with a uniform seeding amplification assay

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Abstract

Currently, early diagnosis of dementia with Lewy bodies (DLB) is based on clinical criteria, which is challenging due to overlapping symptoms with other neurodegenerative diseases. Seeding amplification assays, detecting minute amounts of disease causing α-synuclein (αSyn^D), are emerging as a promising diagnostic tool for α-synucleinopathies including DLB and Parkinson's disease. This study aimed to test whether the same seeding amplification assay established for αSyn^D detection in cerebrospinal fluid (CSF) could be applied to other biospecimens, including skin, olfactory mucosa, saliva, and urine, obtained from the same patients. A total of 31 patients with probable DLB and 53 healthy controls were recruited. When evaluating the assays' applicability to different biospecimens, only those collected from participants with a positive CSF αSyn^D result were considered. Seeding amplification assay results were evaluated based on the αSyn^D amplification rate over 48 h and the value of the area under the curve. The sensitivity and specificity were 94% and 98% for skin, 47% and 100% for olfactory mucosa, and 22% and 100% for urine, respectively for the CSF positive DLB and healthy controls. αSyn^D was undetectable in saliva. Cohen's Kappa analysis (κ) showed almost perfect agreement between CSF and skin assays ($\kappa = 0.86$) but slight to no agreement for CSF versus olfactory mucosa ($\kappa = 0.12$) and urine ($\kappa = 0.094$). In summary, the seeding amplification assay established for αSyn^D detection in CSF demonstrated comparable diagnostic performance in minimally invasive skin biopsies. Olfactory mucosa, saliva, and urine sample preparation pose technical challenges resulting in the established assays' low diagnostic accuracy, for now, limiting their use in diagnostics. Nevertheless, the proof-of-concept for αSyn^D detection in urine expands the potential for non-invasive diagnostics of α-synucleinopathies in the future.

Keywords Real-time quaking-induced conversion, RT-QuIC, Amplification assay, Neurodegenerative, Lewy body, Skin, Cerebrospinal fluid, Olfactory mucosa, Urine

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Introduction

Driven by demographic changes and increased life expectancy, dementia prevalence is rising. Dementia with Lewy bodies (DLB) is the second most common neurodegenerative dementia after Alzheimer's disease (AD). It accounts for 4–8% of diagnosed cases but is assumed to be underdiagnosed [39]. DLB is clinically defined by the McKeith criteria [28], yet misdiagnosis can happen especially in the early disease stages [13, 42] making accurate diagnostic tests necessary.

Neuronal alpha-synucleinopathies such as DLB and Parkinson´s disease (PD) are characterized by the disease causing aggregation of alpha-synuclein (αSyn^D), called Lewy bodies, in the central nervous system (CNS) and peripheral tissues [26].

Besides dementia and parkinsonism, hallmark features of DLB and PD include REM sleep behavior disorder (RBD) and visual hallucinations, which are often preceded by a prodromal phase with hyposmia [11, 37, 46]. Pathologically, Lewy bodies are detected early in the olfactory bulb [26], and the gut, both of which are hypothesized to serve as entry sites for the prion-like propagation of αSyn^D from the periphery to CNS as alternative to CNS-first theory [9, 10, 41].

Seeding amplification assays (SAA) such as Real-Time Quaking-Induced Conversion (RT-QuIC) and Protein Misfolding Cyclic Amplification (PMCA) have emerged as highly sensitive and specific assays for detecting αSyn^D aggregates. αSyn^D SAA exploits the prion-like properties of αSyn^D to amplify minute amounts of misfolded protein, enabling its detection through a fluorescence dye [18]. αSyn^D RT-QuIC is highly specific on both PD, DLB, and RBD but is less sensitive for αSyn^D seeds in multiple system atrophy [7, 38, 40]. In a meta-analysis, PD and DLB could not be discriminated by measurement of αSyn^D using RT-QuIC [36], but there are some reports of DLB having a slightly higher amplification rate [5, 15].

Cerebrospinal fluid (CSF) is a well-established source for biomarker discovery in neurodegenerative diseases and several research groups have demonstrated RT-QuIC to be highly capable of identifying neuropathologically confirmed DLB cases with sensitivity and specificity close to 100% [36]. CSF can thus be considered a golden standard for RT-QuIC in detecting αSyn^D. Recently, the US Food & Drug Administration encouraged the use of αSyn^D SAA for patient screening in clinical trials [17].

However, CSF sampling is invasive and may not be feasible for all patients or for screening protocols, and therefore there is a need for reliable and minimally invasive biomarkers that can enhance diagnostic accuracy in the early stages of DLB and PD, as well as in their prodromal phases. Such biomarkers could play a critical role in identifying candidates for potential disease-modifying therapies. Therefore, other biospecimens like skin [18, 19, 21, 24, 25, 49], olfactory mucosa (OM) [11, 20, 37], and saliva [22, 48, 50] have been tested as alternatives to CSF.

Skin biopsies offer a convenient and minimally invasive method for obtaining peripheral tissue samples. Multiple studies [18, 19, 21, 24, 25, 49] have demonstrated the presence of αSyn^D aggregates in skin biopsies from DLB and PD patients with RT-QuIC, with sensitivity and specificity typically of > 90% and > 85%. In a recen meta-anaysis, skin αSyn^D RT-QuIC has also shown promising results, which almost parallel the sensitivity and specificity reported with CSF samples [52].

The olfactory bulb is one of the first regions affected by Lewy body pathology [2, 10, 26], making OM a promising target for confirming prodromal α-synucleinopathies. Studies have shown that RT-QuIC can detect αSyn^D by nasal swab brushings in DLB/PD/RBD but the sensitivity varies substantially from 48 to 81% [20, 37, 46].

RT-QuIC has also been adapted successfully to detect αSyn^D in saliva with a sensitivity in PD of 84–90% and a specificity versus healthy controls (HC) of 83–92%, potentially offering an entirely non-invasive and easily accessible diagnostic biomarker [22, 48].

Urine has not yet been tested for αSyn^D detection using SAA in alpha-synucleinopathies. However, a study has shown that urine is a suitable material for the detection of variant Creutzfeldt-Jakob's disease, using the PMCA approach with a sensitivity of 93% and 100% specificity [30].

In this study, we aimed to expand the established αSyn^D CSF RT-QuIC assay protocol to multiple biospecimens which include skin, OM, saliva, and urine - all collected from a single cohort. The cohort consisted of patients with DLB, who were positive in RT-QuIC for αSyn^D in CSF, and HC. We describe the optimized preparation of these different biospecimens, report the sensitivity and specificity achieved for each biospecimen using a uniform αSyn^D RT-QuIC protocol with a rapid result, and discuss the opportunities and challenges these biospecimens present for improving clinical diagnostics.

Materials and methods

Study design and ethics

Patients fulfilling the consensus criteria for probable DLB [28] or probable mild cognitive impairment with Lewy bodies (MCI-LB) [29] with Mini-Mental State Examination (MMSE) > 18 were included (from now on both annotated as DLB).

The HC cohort consisted of samples collected from individuals in two groups: (1) Young HCs (yHC); age < 40 years, MMSE score indicative of no cognitive impairment with no first-degree relatives with PD/DLB, and (2) Older HCs (oHC); age 55–75 years with a normal sense of smell defined by Sniffin´ Stick Identification test 16 (a test using 16 different smells, that quantitatively assesses

olfaction adopted to a Danish population [32]) over the 25th age-adjusted percentile [33].

This study is an unblinded single-center cross-sectional case-control study with patients with DLB versus HC. In total, the study cohort consisted of 84 individuals, including 31 clinically diagnosed with probable DLB, 5 yHC, and 48 oHC. CSF sample collection was possible for 22 patients with DLB. Using the established RT-QuIC, all CSF samples were screened for αSyn^D. Only biospecimens from patients with a positive CSF αSyn^D were used for RT-QuIC sensitivity and specificity calculations in different biospecimens. See Table 1 for an overview of patients' demographics, clinical tests, and sample collection. The αSyn^D RT-QuIC data for the whole cohort is available in Additional file 1.

All patients with DLB were recruited within three years of diagnosis. The participants with DLB were older than oHC and included significantly fewer females, see Table 1. Around half of the DLB patients had significant co-pathology of both amyloid-β42 and/or p-tau in CSF. In the CSF SAA negative group, 3 (75%) were p-tau positive compared to the CSF SAA positive group where only 5 (28%) were positive ($p = 0.076$).

Reagents

All reagents used for the collection and preparation of samples, and for αSyn^D RT-QuIC protocol were from Sigma Aldrich® unless otherwise specified.

Collection and preparation of samples

Brain

Brain samples acquired from the biobank of the Danish Reference Center for Prion diseases, with neuropathologically confirmed Lewy body deposits, were used as a standard αSyn^D RT-QuIC positive control. These samples were prepared using fresh frozen frontal cortex tissue collected during cranial autopsy and stored at -80 °C. The presence of Lewy body deposits was confirmed by immunohistochemistry on adjacent formalin-fixed brain tissue samples.

The brain DLB controls were prepared as a 10% *w/v* (10^{-1}) brain homogenate (BH) solution through chemical lysis using tris-buffered saline x 1 at pH 7.4, containing 0.5% sodium deoxycholate and 0.5% tergitol. The BH solution was then transferred to M-tubes™ compatible with the Dispomix™ cell dissociator (Xiril®). Mechanical lysis was conducted at 4000 rpm for 15 s. The resulting BH supernatant was cleared by centrifugation at 800 g for 5 min at 4 °C and diluted a millionfold (10^{-6}) in 1:10 serial dilution in DEPC-treated water.

Cerebrospinal fluid

CSF samples were collected from patients with DLB by lumbar puncture and cleared by centrifugation at 2000 g, 4 °C, 10 min. The cleared CSF samples were aliquoted into 250 µL in polypropylene (PP) tubes and subsequently stored at -80 °C until use. CSF samples were discarded if the total protein count was > 1 g/L, erythrocyte count > 300, and white blood cell count > 5. CSF samples were less than two years old when tested in RT-QuIC.

Table 1 Demographics and biospecimen collection of the study cohort

	Total DLB cohort	DLB CSF SAA+	DLB CSF SAA-	yHC	oHC	All HC
N	31 (3 MCI)	18 (3 MCI)	4	5	48	53
Age, mean (SD)	75.0 (5.9)	74.5 (4.2)	74.5 (9.5)	31.2 (4.8)	67.5 (5.1)	64.1 (11.9)
Sex, N female (%)	4 (12%)	1 (6%)	0	2 (40%)	28 (58%)	30 (57%)
MoCA, mean (SD)	27 (2.0)	21 (4.4)	18.2 (4.3)	29.6 (1.2)*	27 (2.0)	NA
β-amyloid-42, N Positive (%)	16 (67%)	11 (61%)	3 (75%)	NA	NA	NA
P-tau, N Positive (%)	10 (42%)	5 (28%)	3 (75%)[a]	NA	NA	NA
CSF, N	22	18	4	0	0	0
Skin, N	31	18	4	5	47	52
Olfactory Mucosa Swab 1, N	30#	17¤	4	4	48	52
Olfactory Mucosa Swab 2; N	17	8	3	1	37	39
Saliva, N	31	11	4	5	NA	5
Urine, N	29	18	4	4	8	12

Table showing the demographics, cognitive status and markers for co-pathology in the total cohort of DLB, the DLB with CSF available (positive and negative), and healthy controls (young and old). Note that not all DLB (31) had CSF available for SAA testing. Generally, swab 1 was treated with method 1 and swab 2 with method 2, except all oHC swabs were treated with method 2

Abbreviations: CSF = cerebrospinal fluid, CI = 95 % Confidence intervals, DLB = Dementia with Lewy body, MoCA = Montreal Cognitive Assessment, N = number of samples, NA = not applicable, OM = olfactory mucosa, SAA = seeding amplification assay, SD = standard deviation, oHC = older healthy controls, yHC = young healthy controls

* MMSE score

[a] Difference between SAA+/- DLB $p = 0.076$

3 DLB swabs were discarded due to no pellet

¤ 2 DLB swabs discarded due to no pellet

The CSF samples were used immediately after thawing and without any processing. Freeze/thaw cycles were noted and did not exceed more than three times [1].

Olfactory mucosa

OM samples were collected via nasal endoscopy in both nostrils via swabbing from the agger nasi area and anterosuperior part of the middle turbinate bilaterally using either nylon (FLOQswabs™ Copan® Diagnostics) (N of oHC = 48) or fiber-based swabs (Nasopharyngeal Specimen Collection Swab, NEST®) (N of DLB = 30, and yHC = 5). Topical anesthesia with Lidocaine and Epinephrine (20 mg + 6,7 ug/mL) was administered using cotton gauze placed towards the middle turbinate. The gauze remained in place for at least two minutes.

Two final-year medical students were trained in the procedure by a rhinologist. The rhinologist was consulted for supervision in case of uncertainty. For patients with DLB and yHC, a maximum of two samples were collected, one from each side. For oHC, a maximum of four swabs were collected, two from each side. In certain cases, fewer samples were obtained if not technically feasible. Collected swabs were submerged into 15 mL PP tubes containing 3 mL saline solution at pH 7.4. Blood contamination was noted.

The technical difficulty of the sampling procedure was scored on a five-point scale based on the following variables: Anatomical recognizability, patient tolerance, and certainty of correct placement of swabs on each side. A score of zero indicated that the procedure could not be performed, while a score of five points meant flawless execution without any issues or uncertainties.

Tubes with swabs were vortexed separately at a speed of 18,0000 rounds per minute for 1 min., followed by a second vortex with a combination of all swabs in a new tube. Sample solutions were pelleted at 2000 g (DLB and yHC) and 550 g (oHC), 4 °C, 20 min to yield a visible cell pellet (off-white to custard yellow). Cell pellets in solution were stored at -80 °C until the lysis protocol initiation. Bloody pellet samples were discarded.

To confirm the presence of olfactory mucosa in the nasal swabs, we performed a cytological spot check of 19 random samples. For the description of this method see Additional file 1.

The OM samples were prepared using two extraction methods. In method 1, a thawed cell pellet from a single swab was suspended in 0.075% sodium dodecyl laurate (SDS) in phosphate-buffered saline (PBS) x 1 at pH 7.4. To the suspension were added 800-micron silica beads (OPSDiagnostics®) and vortexed at maximum speed for 30 s. All 30 DLB OM samples were first processed using method (1) Method 2 was inspired and instructed by M. Bongianni et al. with few modifications [8]. OM cell pellets were thawed, and a sterile 10 µg inoculating loop (Sarstedt®) was used to transfer one loop of cells to 100 µL 40 mM sodium phosphate buffer (ThermoFisher®), pH 8. The cell suspension was subjected to cell dissociation in an ultrasonic water bath (Emag® EMMI, 120 W) at 100% ntensity until total dissociation (~ 30 s) of the pellet. Overall, 17 out of 30 DLB OM samples were processed with both methods 1 and 2, while 13 DLB OM were only processed with method 1 due to lack of material (Additional file 1). All oHC OM were processed with method (2) The same RT-QuIC script was applied for both methods.

Skin biopsy

Skin biopsy samples were collected from the neck region (C7) five cm paramedially after administration of local anesthetic and adrenaline. Two biopsies were taken from each participant, each measuring 3 mm. The biopsies were dabbed for blood on a cotton cloth, thoroughly washed with cold PBS x 1 at pH 7.4, and stored dry at -80 °C until lysis protocol. For participants with DLB and yHC, one skin biopsy was used for RT-QuIC, whereas for oHC, both biopsies were used.

The skin biopsies were prepared as a 5% *w/v* stock solution by chemical lysis using PBS x 1 at pH 7.4, 1% triton x-100, 1 x total protease cocktail inhibitor (Promega®), and 150 mM NaCl. The solution containing the biopsy was transferred to M-tubes™ compatible with the Dispomix™ cell dissociator (Xiril®). Mechanical lysis was conducted by a 30-second interval program increasing from 1100 rpm to 4000 rpm. The resulting supernatant was cleared by centrifugation at 800 g for 5 min at 4 °C. The 5% *w/v* stock solution was further diluted to a 1:10 (10^{-2}) solution in DEPC-treated water.

Saliva

Saliva samples were collected after participants adhered to a minimum fasting period of 1 h, abstaining from food, beverages, and smoking (water excepted). Samples were collected directly into PP tubes and kept on ice until centrifuged at 2000 g for a minimum of 10 min, at 4 °C. The supernatant was aliquoted and stored at -80 °C until use.

The saliva samples were processed in multiple ways which are listed in Additional file 1.

Urine

Urine samples were collected by urination into a 100 ml PP beaker. There were no requirements for the sample material and no restrictions from the last urination. The content was transferred to 50 ml PP tubes and stored at -80 °C.

After thawing 15 ml urine was transferred to a suitable-sized spin filter (sartorius®) with a molecular weight cut-off of 100 kilo Daltons. Filters were centrifuged at 3200 g, 30 min at 4 °C until filter dead volume (< 100 µL). The

dead volume was dissolved into a total of 500 µL 40 mM phosphate buffer which resulted in a 30x concentrated sample. The resulting solution was used to flush the filter before transferring it to a microcentrifuge tube.

αSyn^{D} RT-QuIC

Production of recombinant αSyn

The production of recombinant αSyn (rec-αSyn) substrate with an N-terminal 6x-histidine-tag was optimized from a previously reported protocol [14]. A 5 mL liquid culture of Lysogeny Broth media was inoculated from a glycerol stock of transformed E. coli until it reached an OD600 of 0.3–0.5. The culture was then diluted 1:1000 in Overnight Express Terrific Broth (OETB™, Novagen®) media and cultivated in 2 L baffled flasks at 30 °C, 180 rpm, overnight. Cells were pelleted at 4000 g for 15 min and carefully resuspended in 25 mL osmotic shock buffer (40% sucrose, 2 mM EDTA, 30 mM Tris, pH 7.2) per 250 mL of culture media. The suspension was stirred in rotation for 10 min at room temperature and pelleted at > 7000 g for 20 min at 20 °C. The supernatant was discarded, and each wet pellet was resuspended in 10 mL ice-cold 20 mM Tris-Hydrochloric acid (HCl) buffer supplemented with 20 mM imidazole, pH 7.5. Each pellet was sonicated on ice (Sonifier SFX150, microtip) at 70% intensity for 30 s over two rounds with a 10-second pause in between. The resulting suspension was stirred in rotation for 10 min at 4 °C and pelleted at > 7000 g for 30 min at 4 °C. Pellets were discarded, and the supernatant was acidified to ~ pH 3.5 with 1 M HCl. The solution, along with the resulting precipitate, was centrifuged at 7000 g for 30 min at 4 °C, and the pH was elevated to ~ 7.5 with 1 M NaOH. The solution was sterile filtered (0.45 µm) and loaded (20 mM imidazole, 20 mM Tris, pH 7.5) onto a 5 mL prepacked HisTrap™ HP column (Cytiva®). The column was washed with 50 mM imidazole, pH 7.5. The protein was eluted in a linear gradient with 15 column volumes of 20 mM Tris, 500 mM imidazole, pH 7.5. The eluate was sterile filtered (0.22 µm) and dialyzed overnight in 40 mM sodium phosphate buffer, pH 8 (ThermoFisher®). The dialysis buffer was exchanged, and dialysis continued for a further 4 h. Protein concentration was estimated with the Qubit® protein assay kit. Each production batch was labeled with a batch number and validated by an RT-QuIC test run. Best yield was 60 mg rec-αSyn pr. 1 L culture.

RT-QuIC set-up

The αSyn^{D} RT-QuIC assay was conducted using a FLUOStar™ Omega instrument from BMG LABTECH®, with a black 96-well plate featuring a clear flat bottom (Greiner®). Each well contained six 800-micron silica beads (OPSDiagnostics®). The total reaction volume per well was 100 µL, including the sample and master mix comprised of 40 mM phosphate buffer (ThermoFisher®) at pH 8.0, 170 mM NaCl, 10 µM Thioflavin T, 0.0015% SDS in PBS x 1, and 0.1 µg/µL of freshly thawed rec-αSyn. CSF samples were added in volumes of 7 and 15 µL. Processed BH, skin, and OM samples were added in 2 µL volumes. Urine samples were added in 15 µL volumes, while saliva samples were added in volumes ranging from 1 to 10 µL, Additional file 1. The plate was sealed with adhesive film (MicroAmp®) and subjected to alternating intervals of 60 s of double orbital shaking at 400 rpm and 60 s of resting, continuously throughout the assay duration. Fluorescence measurements were taken every 45 min for a total of 48 h, expressed in Relative Fluorescence Units (RFU), at 450 ± 10 nm excitation and 480 ± 10 nm emission.

RT-QuIC interpretation

αSyn^{D} RT-QuIC assay experiments were performed in quadruple wells (referred to as replicates) for each volume. The threshold for a positive replicate was set at 25.000 RFU (4 standard deviations from the Thioflavin T background RFU), and any given sample and control were considered positive if ≥ 2 of 4 replicates crossed the threshold within 48 h. For samples with > 2 positive replicates, the two best-performing replicates were used for further analysis to ensure an optimal comparison of seeding activity between samples. For samples with < 2 positive replicates (deemed negative), the mean of all four was used for further analysis, to ensure that the variance of the single positive replicates was transparent in the data visualization (Fig. 1, f). We included the single positive replicates in the plot (Fig. 1, f) to demonstrate the robustness of the assay towards the healthy control cohort when pooled together.

The kinetic performance of RT-QuIC is interpreted based on the increase of RFU over time. The performance progress can be divided into a lagging phase (lag-phase), a logarithmic phase (log-phase), and a stationary phase (plateau in RFU) which are all quantified by the Area Under Curve (AUC). Additionally, maximum RFU, lag-phase time, final RFU and time to half the maximum RFU (TH50) are also calculated. DLB sample performance is evaluated by the shortest lag-phase, broadest log-phase, and earliest stationary phase, while HC is expected to have a constant lag-phase under the set RFU threshold.

Statistics

The visualization of results was performed using GraphPad Prism 10. RFU over time displaying the kinetic development is presented as the mean RFU with accompanying 95% confidence intervals (CI). The AUC was computed with the software's default parameters and comparisons of AUC values across groups were performed using a one-way analysis of variance (ANOVA).

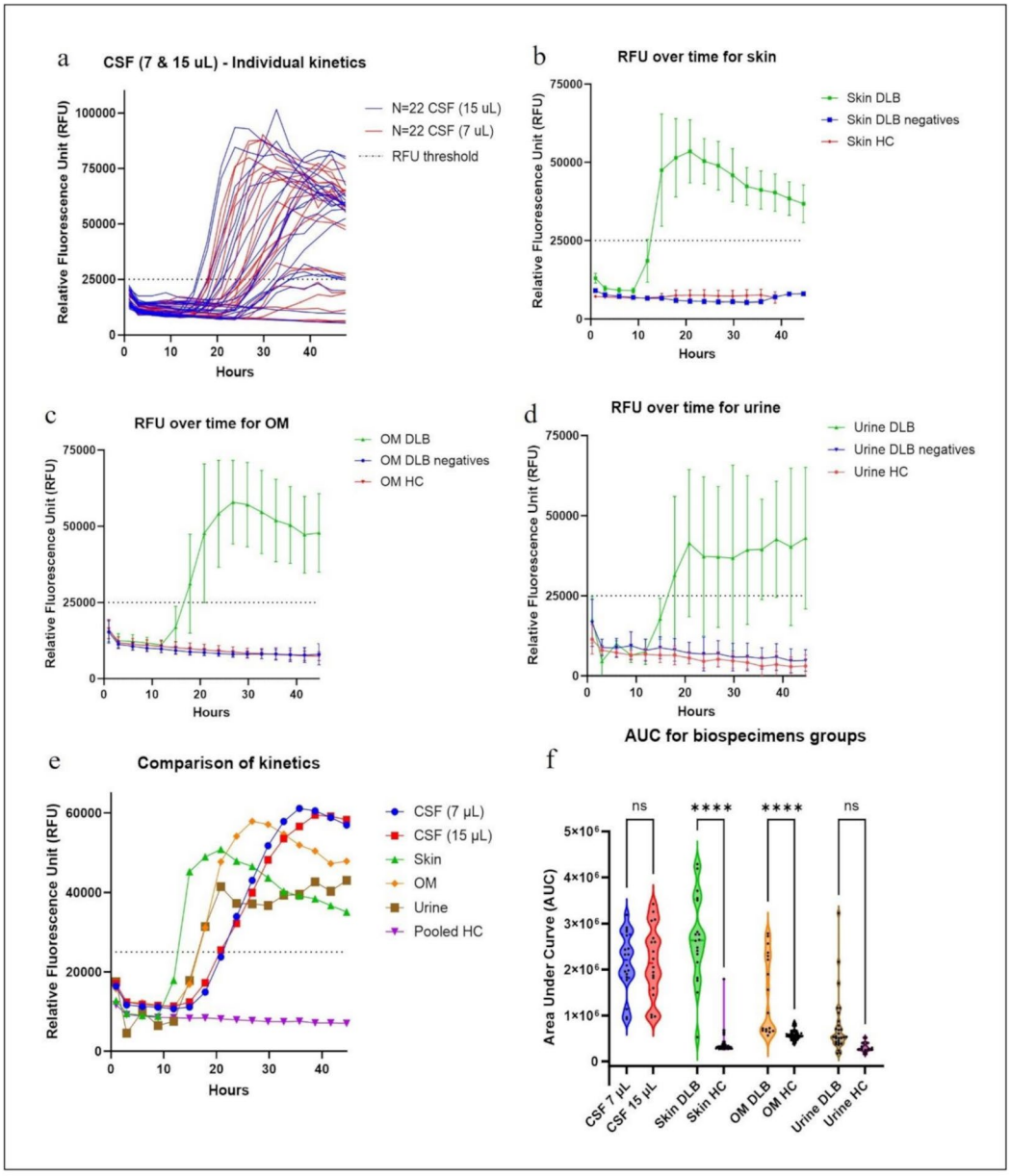

Fig. 1 Kinetics and AUC for each biospecimen group. (**a**) Individual curves for all CSF samples at CSF volumes 7 µL and 15 µL (**b**) RFU over time for skin groups (**c**) RFU over time for OM groups (**d**) RFU over time for urine groups (**e**) Comparison in kinetics between biospecimens (**f**) Area Under Curve (AUC) for the biospecimen groups. Each black dot is a patient sample, and the violin plot displays the distribution

Sensitivity, specificity, and diagnostic accuracy were calculated in R (version 4.3.3) and reported with CI using Wilson's method in the *binom* package. The agreements between biospecimens were calculated using the *psych* package for Cohen´s Kappa (κ) analysis, with Cohen´s interpretation: below 0 = No agreement, 0.01–0.2 = None to slight agreement, 0.21–0.39 = Fair agreement, 0.40–0.59 = Moderate agreement, 0.60–0.79 = Substantial agreement, above 0.80 = Almost perfect agreement [27].

Results

αSyn^D RT-QuIC sensitivity and specificity for individual groups of biospecimens

Twenty-two CSF samples were analyzed for the presence of αSyn^D at 7 µL and 15 µL CSF volumes per reaction and the kinetic parameters were compared (Fig. 1, a). The CSF sensitivity was calculated based on agreement with clinical diagnosis. Out of the 22 samples; 18 samples were positive at 15 µL and 17 samples were positive at 7 µL. The sensitivity at 15 µL and 7 µL CSF volumes were 82% (CI = 61–93%) and 77% (CI = 57–90%), respectively.

Table 2 The sensitivity, specificity, accuracy and kinetic parameters of biospecimens from CSF SAA-positive DLB versus HC

	Sensitivity % (CI)	Specificity % (CI)	Accuracy % (CI)	MaxRFU (CI)	AUC (CI)	Lag-phase (h)	Final RFU (48 h)	T50 (h)
Skin	94 (74–99)	98 (90–100)	96 (90–100)	54,894 (31176–39556)	1.56×10^6 ($1.33–1.79\times10^6$)	12	35,547	13
Olfactory Mucosa combined	47 (26–69)	100 (93–100)	74 (63–84)	52,200 (30894–38933)	1.65×10^6 ($1.38–1.92\times10^6$)	15	46,603	17
Olfactory Mucosa Swab 1	24 (10–47)	100 (93–100)	62 (52–71)	-	-	-	-	
Olfactory Mucosa Swab 2	62 (31–86)	100 (91–100)	81 (67–95)	-	-	-	-	
Urine	22 (9–45)	100 (76–100)	61 (50–72)	48,375 (25866–33385)	1.39×10^6 ($1.08\times1.79\times10^6$)	15	48,375	17

The sensitivity, specificity, accuracy, Maximum RFU, AUC, hours of lag-phase, final RFU and TH50 of the biospecimens calculated only for the SAA CSF positive DLB samples. Abbreviations: CI = 95% Confidence Interval, MaxRFU: Maximum RFU, h: hours, T50: Time to reach 50% of maximum RFU

The sensitivity, specificity, diagnostic accuracy and kinetic parameters were calculated for skin, OM, and urine to evaluate the efficiency of the αSyn^D RT-QuIC in discriminating between the DLB CSF positive and HC groups (Table 2). The RT-QuIC performed excellently for the skin group (Fig. 1, b), with a diagnostic accuracy of 96% (CI. = 90–100%) for CSF SAA positive DLB versus HC.

The OM samples preparation (but not the SAA parameters) was optimized from method 1 to method 2, increasing the sensitivity from 24 to 62% without changing the specificity. The combined swabs resulted in a final diagnostic accuracy of 74% (CI = 63–84%) (Table 2; Fig. 1, c). There was a broad gap between the diagnostic accuracies when comparing OM samples processed solely by either method 1 or method 2. Increasing the assay time for OM up to 80 h did not change the outcome of the result.

Lastly, the urine group demonstrates a low αSyn^D detection, with a sensitivity of 22% and with a lack of reproducibility in 3 out of 5 samples (Fig. 1, d). It was noted that the urine samples had significant precipitations, and this was seen for every freeze and thaw cycle. The urine HC group was negative, resulting in a 100% specificity, indicating that there is not a common tendency for false positivity across urine samples using this method.

When comparing the Cohen's agreement of DLB CSF RT-QuIC versus skin RT-QuIC results the agreement was almost perfect, $\kappa = 0.86$, CI 0.6–1), whereas CSF versus OM (combined method) had a none to slight agreement, $\kappa = 0.12$, CI -0.17–0.42), and CSF versus urine had a none to slight agreement, $\kappa = 0.094$ (CI -0.027–0.22). The agreement was higher when comparing skin (DLB and HC) to OM (combined), $\kappa = 0.52$ (CI 0.31–0.72, moderate agreement), and skin to urine, $\kappa = 0.19$ (CI 0.028–0.35, none to slight agreement). The agreement between OM methods 1 and 2 was only fair ($\kappa = 0.34$, CI -0.062–0.75).

When regarding the entire DLB cohort (which includes DLB patients that were negative in CSF RT-QuIC), the sensitivities were 77% (CI = 60–89%) for skin, 40% (CI = 25–58%) for OM, and 17% (CI = 8–35%) for urine (Additional Table S1).

Multiple approaches were tried on saliva, both in sample preparation and to adjustments of the RT-QuIC set-up, but no detection of αSyn^D was achieved. We suspected that the lack of amplification of potentially present αSyn^D is due to strong suppressive effects from the saliva matrix. Therefor we tested the effect of the saliva matrix by spiking HC saliva with DLB BH (10^{-2} to 10^{-5}), which demonstrated a strong suppressive effect, with only the 10^{-2} dilution (10^4-fold concentrated compared to the standard DLB BH control) being significantly amplified.

OM sampling and cytological spot check

There were no significant differences in the technical grading of the sampling procedures between DLB and oHC (Median (IQR): DLB = 4 (3–5) vs. oHC = 4 (3–5), $p = 0.8$). The cytological spot check of 19 samples revealed that three swabs (16%) either had no cells or cells with morphology that could not be studied, while the 16 swabs (84%) were positive for neuronal markers, which are specific for the olfactory mucosa but absent in the additional nasal mucosa, Additional file 1, Fig. S1).

Comparison of αSyn^D RT-QuIC AUC and kinetics across biospecimen groups

The best-performing biospecimens, ranked by highest mean AUC values, are CSF 7 µL, CSF 15 µL, OM, skin, and urine, respectively. However, the difference in mean AUC of the positive samples (Fig. 1, f) between biospecimen groups is minimal. There was no significant difference in AUC values (Fig. 1, f) when comparing 7 µL and 15 µL, and no correlation in a linear model between maximum RFU or AUC with MoCA (data no shown). When taking the best performing volume (CSF

7 µL) as a reference, the mean AUC difference in percentage is 2.3%, 2.7%, and 5.5% for CSF 15 µL, OM, and skin, respectively. This demonstrates an identical AUC pattern between biospecimens in the assay set-up. When comparing the lag-phase duration for all biospecimens (Fig. 1, e), we observe an early stationary phase within 16 to 36 h, regardless of sensitivity. The skin samples are the first to reach the stationary phase, followed by OM, and lastly CSF. When evaluating best-performing biospecimens on the shortest lag-phase duration, we observe the opposite trend than observed for AUC with the best being skin, OM, and CSF, respectively.

Lastly, we evaluated the number of positive replicates (quadruple set-up) for each positive sample. We found that every replicate was positive (100%) for each positive CSF and skin sample, and for OM and urine, the positive replicate rates were 95% and 40%, respectively.

Discussion

We aimed to test an established CSF αSyn^D RT-QuIC assay for its application to screen multiple biospecimens for the presence of αSyn^D without altering the assay protocol, and to compare their efficiency to CSF. We demonstrate that the described αSyn^D RT-QuIC can detect αSyn^D from multiple minimally invasive biospecimens. Our results on skin biopsy samples for αSyn^D RT-QuIC demonstrate a diagnostic accuracy of 96%, similar to other studies [19, 24] and a κ of 0.86, confirming the validity of using skin biopsies for the detection of αSyn^D instead of the more invasive CSF. The sensitivities for detecting αSyn^D in OM and urine were moderate to low in comparison to CSF and skin, which we primarily attribute to obstacles in sampling and processing techniques.

The OM varied in viscosity and amount of cell material, and these variations can certainly influence the outcome of the result. Less variation such as biopsy weight were noticeable in skin processing and total homogenization, and thereby lowering the failure risk. We compared the AUC of each biospecimen group and observed that the early stationary phase is not correlated to a higher AUC, as the stationary phase tends to drop over time (Table 2). Skin had the most rapid amplification with a lag phase of 12 h, and this can be attributed to skin samples being lysed with a buffer containing 1% Triton X-100 (with a final concentration of 0.002% Triton X-100 in the RT-QuIC assay well), which has been proven to accelerate αSyn^D amplification [24]. The prolonged lag phase of CSF (18 h) compared to the other biospecimens is likely due to minor inhibitory effects exhibited by CSF [45]. This inhibitory effect may also explain why CSF at 7 µL (Fig. 1, a, blue lines) performs slightly better than at 15 µL (Fig. 1, a, red lines), even though the αSyn^D seeding should theoretically be higher. Despite the observed differences in kinetics between biospecimens, arising from the nature of the samples or processing technique, we still observe an overall similarity. This similarity demonstrates that the SAA methodology plays a crucial role in the kinetic development and altering the SAA methodology would likely alter the kinetics as well. This is significant regarding what these kinetics can actually tell us about the patient. The reported performance of αSyn^D RT-QuIC on OM samples has varied in previous studies, with sensitivities ranging from 46% [46], 48% [20] to 56% [11] for PD and 83% for DLB [8]. The variance could partly be explained by different groups (PD, RBD, and DLB) but the sampling and further processing could also be important aspects. When we changed the OM extraction method to the described method by Bongianni M. et. al. [8] we achieved a doubling in sensitivity without any other alterations, e.g. the RT-QuIC script. Unfortunately, we did not have sufficient swabs to perform method 2 on all the swabs in the DLB group. The increased sensitivity is likely due to method 2 being more efficient in releasing intracellular material and assessing the pellet for aliquoting, and is therefore our recommended method. To assess the quality of the OM sampling material we randomly selected 19 nasal swabs for cytology spot check and 16% of these were not positve for neurons specific for olfactory mucosa. Even though the cytology spot checks were performed on a small number of samples it demonstrates the challenge of successful sampling, which could be a partial explanation for the lower sensitivity. Perhaps, a cytology spot check would be a standard test along with the SAA result to validate proper sampling. We sought to overcome the risk of incorrect sample material by using two swabs.

Previous studies on OM [11, 20, 37] with varied sensitivities were all performed on RT-QuIC assay parameters that differed (primarily a longer rest cycle time of 14 and 29 min). This results in a prolonged lag-phase of + 30 h and with an assay time of + 70 h, when compared to assays using short resting cycles (~ 1 min) requiring only 48 h at maximum [5, 24]. Our assay on OM is therefore, to our knowledge, the most rapid with a lag-phase of only 16 h and a stationary phase reached at 24 h.

We have investigated the use of saliva samples for αSyn^D detection but did not achieve a successful result, despite numerous efforts. The strategies were aimed at overcoming a strong suppressive effect from the biological material. This suppressive effect was clearly observed when we seeded HC saliva samples with our DLB BH control at dilutions ranging from 10^{-3} to 10^{-6}, inspired by Kuzkina A. et al., who investigated a suppressive effect in OM samples, collected from nasal brushings [20]. Luan M. et al. have reported the successful detection of αSyn^D in saliva samples from PD patients [22, 23]. Of notice, the reported assay parameters are different from ours, especially in the amount of added rec-αSyn pr well, which is

ten-fold higher. We have tried multiple assay modifications but have not substantially increased the rec-αSyn. Another published successful approach is to combine SAA with immuno-precipitation (IP) strategies [50] to isolate present αSyn species.

We report the first proof-of-concept αSyn^D SAA detection in minimally processed urine samples. With thorough optimization, urine may have the potential to become a new biospecimen suitable for detecting αSyn^D in patients with α-synucleinopathies. The lack of reproducibility for 3 out of the initial 5 positive DLB samples is primarily attributed to the possible impact of freezing and thawing urine samples. Saetun et al. demonstrated that the freezing and thawing of urine samples reduced the presence of urinary proteins [43]. The specificity for urine was 100% and we therefore do not attribute the initial positive results to be false. Furthermore, requirements such as urination restriction prior to sampling could be considered. In this study, such requirements were not applied due to a high presence of urge incontinence in participants with DLB. The freezing of urine samples could also be avoided totally by diluting urine samples in a preservation medium (i.e. phosphate buffer supplemented with protease inhibitor cocktail), which could keep samples stable at 4 °C, prior to sample concentration. To our best knowledge, the depositions of αSyn^D in urine have not yet been explored for αSyn^D detection with SAA, but Nam D. et al. [31] reported utilizing urine samples for detecting abnormal levels of αSyn^D in PD patients using an Enzyme-Linked Immunosorbent Assay based method.

Although SAA exhibited a high combined sensitivity (81%), six participants diagnosed with probable DLB remained negative in all biospecimens (Table S1). Given the absence of neuropathological confirmation and the limited sample size, it remains uncertain whether these DLB cases represent phenocopies of other pathologies or if they are false negatives attributed to SAA testing. It is worth noting that there was a trend for higher p-tau pathology in the SAA negative CSF versus SAA positive (Table 1). A study of αSyn^D copathology in AD found a negative correlation between tau and αSyn^D SAA positivity [44]. Weather the increased p-tau represent a different pathology e.g. AD or tau have an inhibitory effect on the SAA cannot be determined from this study. Furthermore, recent studies have reported reduced sensitivity of SAA for Lewy body pathology confined to the olfactory bulb/brainstem [6, 16, 44], amygdala-predominant [3, 16, 44, 47], and limbic system [47]. Consequently, future research involving larger, clinically more divers cohorts with available neuropathological analyses may clarify the issue of SAA-negative DLB cases.

The RT-QuIC sensitivity of the total DLB cohort (Additional Table S1) is lower compared to the subgroup identified as positive for CSF SAA (Table 2). This distinction is important to consider when implementing the assay in a typical clinical setting where the cohort has not been prescreened for CSF SAA positivity.

Despite the promising results, there are few limitations in our study design. The study lacked complete sample blinding. However, the RFU threshold applied in this study for positive and negative RT-QuIC results was predefined in advance based on our own preliminary data and published recommendations [10]. The CSF samples collected from patients with clinical DLB were used to screen for the presence of αSyn^D. It is a limitation that we do not have CSF samples from the HC group. However this decision was made to increase the number of voluntiers in the HC group, who were more accepting of other biospecimen collection, contrary to lumbar puncture. Additional limitation regarding the HC group is that this group was younger than the DLB group by 10 years. The lower mean age and higher cognitive score in the HC group has likely contributed to increased specificity in this study. However, it also provides valuable context regarding the technical performance in HC participants, whom we consider true negative controls.

αSyn^D RT-QuIC has advanced since its first report by Fairful G. et al. [12], with over a hundred studies on various biospecimens, RT-QuIC set-ups, and recombinant αSyn substrate types. Initially, RT-QuIC was adopted to diagnose Creutzfeldt-Jakob Disease [4, 35, 51] and a comprehensive ring-trial with definitive samples was later conducted among established laboratories [34], demonstrating that different in-house assay protocols can yield the same diagnostic conclusions. Similar ring-trials are needed on reported αSyn^D RT-QuIC protocols to evaluate their diagnostic potential, and would also help to understand why some biospecimens, like OM and saliva, perform well only in certain laboratories.

Conclusion

We have established a multi-biospecimen αSyn^D RT-QuIC with rapid performance and feasible sample processing techniques. The diagnostic performance of skin is as powerful as CSF with a diagnostic accuracy of 96% (CI = 90–100%), while OM demonstrates moderate accuracy at 74% (CI = 63–84%), and with opportunities for further improvement in sampling and processing. Detection of αSyn^D in saliva samples failed, likely due to the sample material inhibiting the assay performance. Lastly, we have demonstrated the first proof-of-concept detection of αSyn^D in urine with minimal sample processing, potentially bringing another non-invasive strategy into the diagnostic toolbox for α-synucleinopathies.

Abbreviations

AD	Alzheimer's disease
AUC	Area under curve

BH	Brain homogenate
CSF	Cerebrospinal fluid
CNS	Central nervous system
CI	Confidence intervals
DLB	Dementia with lewy bodies
HC	Healthy controls
HCl	Hydrochloric acid
lag-phase	Lagging phase
log-phase	Logarithmic phase
MCI-LB	Mild cognitive impairment with Lewy bodies
MMSE	Mini-mental state examination
MoCA	Montreal cognitive assessment
OM	Olfactory mucosa
oHC	Older HCs
ANOVA	One-way analysis of variance
PD	Parkinson´s disease
PBS	Phosphate-buffered saline
αSyn^{D}	Disease causing α-synuclein
PP	Polypropylene
PMCA	Protein misfolding cyclic amplification
RT-QuIC	Real-time quaking-induced conversion
RFU	Relative fluorescence units
RBD	REM sleep behavior disorder
RBD	Seeding amplification assays
SDS	Sodium dodecyl laurate
yHC	Young HCs

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40478-025-02034-8.

Supplementary Material 1

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Author contributions

Conceptualization was the effort of RB, OHM, GW, SGH, CvB, CKP, KSF, ELL and AA.Writing-original draft was the effort of RB, OHM and AA. AHS, MB, CHP, SOB, JL, PE, MB, EB, GZ, RB, OHM, GW, SGH, CvB, CKP, KSF, ELL and AA contributed to the Methodology and Writing – review & editing. Supervision and funding were the effort of GW, SGH, KSF. ELL and AA.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by The Danish Research Ethics Committee (H-22046053 and H-22053428) and The Danish Data Protection Agency (P-2022-668 and P-2022-669). All patients gave written informed consent to participate in the study.

Consent for publication

All authors have read and approved this manuscript for publication. All patients gave consent for publication of study results.

Competing interests

The authors declare no competing interests.

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