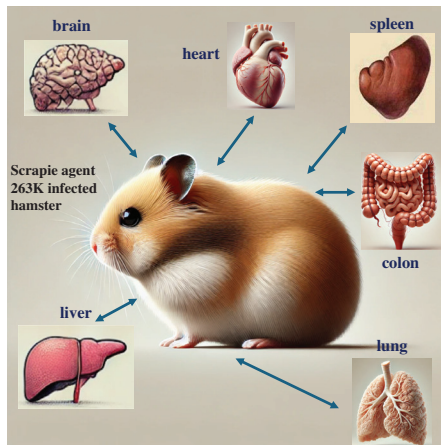




ORIGINAL ARTICLE

RT-QuIC Reactivity in the Brain and Other Organs and Tissues in Early, Middle-early, Middle-late and Terminal Stages of Scrapie Agent 263K Intracerebral Infection in Hamsters

Graphical abstract



Highlights

- The study shows that prion widely distributes in the brain and peripheral tissues of 263K scrapie-infected hamsters. Brain tissue shows stronger RT-QuIC reactivity than peripheral tissues which have delayed increasing positivity. Also, Brain tissue has higher SD_{50} values and shorter lag times than peripheral tissues in which skin tissue shows better value on RT-QuIC test than others.

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Brief statement

The study shows that prion widely distributes in brain and peripheral tissues of 263K-infected hamsters and brain tissue shows stronger RT-QuIC reactivity. Skin tissues are effective for RT-QuIC test and can be used for early prion disease detection.

RT-QuIC Reactivity in the Brain and Other Organs and Tissues in Early, Middle-early, Middle-late and Terminal Stages of Scrapie Agent 263K Intracerebral Infection in Hamsters

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Abstract

Objective: This study was aimed at analyzing the distribution and progression of prion seeds across the central nervous system (CNS) and peripheral tissues, by evaluating the real time quaking-induced conversion (RT-QuIC) reactivity of the brain, and other organs and tissues, from hamsters in early, middle-early, middle-late and terminal stages of intracerebral infection with scrapie agent 263K.

Methods: Brain, skin and other organ specimens were collected at approximately 20, 40, 60 and 80 (terminal stage) days post-inoculation, and homogenates were prepared. Protein misfolding cyclic amplification (PMCA) and RT-QuIC were used for sample detection. The 50% seeding dose (SD_{50}) values for prion seeding were calculated with the Spearman-Kärber method, and RT-QuIC reactivity lag times were compared among tissues and time points.

Results: RT-QuIC indicated positive reactions in brain tissue samples across all time points (20–80 dpi), and increasing seeding capacity and decreasing lag times were observed during the incubation period. In peripheral tissues, including the heart, liver, spleen, lung, colon and skin, RT-QuIC positivity showed delayed onset, appearing at later stages (≥ 40 dpi) than observed in the brain. The brain exhibited significantly higher SD_{50} values than peripheral tissues, thus reflecting greater seeding capacity. Prion seeds were widely distributed in the CNS and peripheral tissues at the terminal stage, and the highest RT-QuIC positivity and SD_{50} values were observed in the brain.

Conclusions: Prions were widely distributed in the CNS and peripheral tissues of scrapie-infected hamsters, and CNS tissues exhibited a particularly high level of reactivity and seeding capacity in the RT-QuIC assay.

Key words: prion, scrapie agent 263K, hamster, RT-QuIC, SD_{50}

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INTRODUCTION

Prion diseases are a group of fatal and transmissible neurodegenerative disorders that affect humans and various animal

species. The pathogenic agent of prion disease is prion or scrapie isoform of the prion protein (PrP^{Sc}), a misfolded conformational variant of the host protein

cellular prion protein (PrP^C). This misfolded protein can self-replicate without detectable nucleic acid involvement [1,2]. Historically, the diagnosis of prion diseases, particularly in humans, has relied on postmortem brain analysis. The development and application of *in vitro* prion amplification techniques, including protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), have enabled prion detection even before clinical disease onset [3,4]. Notably, RT-QuIC has been clinically applied to cerebrospinal fluid and skin samples for the diagnosis of human prion diseases, and has high sensitivity and specificity [5–7].

Prions localize predominantly within central nervous system (CNS) tissues; however, the extent of peripheral organ involvement varies among prion strains and host species. Systematic assessments of the transmission risks associated with various organs and tissues in human and animal prion diseases have indicated a broader distribution of prions in peripheral tissues in sheep and goats with scrapie, and in cattle with bovine spongiform encephalopathy (BSE), than human prion diseases, e.g., sporadic Creutzfeldt-Jacob disease (CJD) [8–10]. Variant CJD, the human form of BSE, exhibits higher prion loads than sporadic CJD in peripheral tissues, including the tonsils, appendix, blood and lymphoid tissue [8,11]. Although prion loads are usually much lower in peripheral tissues than CNS tissues, they nonetheless threaten human and animal health [8,12–15].

Prion-infectious rodent models, whether wild-type or transgenic, are extensively used in studies of prion pathophysiology. Among them, the scrapie strain 263K infected hamster model is widely used, because of its consistent clinical and neuropathological features [16,17]. Herein, we analyzed the RT-QuIC reactivity of organs collected at various time points after intracerebral inoculation with the 263K strain, including brain, heart, liver, spleen, lung, colon and skin tissues. Positive RT-QuIC reactions were detected in all tested tissues at the terminal stage. End-point dilution RT-QuIC assays revealed markedly higher seeding capacity in brain tissues than other peripheral tissues, as evidenced by elevated positivity rates and shorter lag times. The seeding capacity of prion seeds increased during the incubation period. Calculation of 50% seeding dose (SD₅₀) values with the Spearman-Kärber method confirmed the much higher prion titers in brain tissues than other tissues.

MATERIALS AND METHODS

Scrapie 263K infected experimental hamsters

Brain samples from hamsters intracerebrally inoculated with 263K scrapie agent were collected at approximately 20, 40, 60 and 80 (terminal stage) days post-inoculation (dpi). The bioassay procedures, along with the clinical, neuropathological and pathogenic characteristics of these infected animals, have been described previously [18,19]. The average incubation period of 263K-infected hamsters

was 80.1 ± 5.7 days. Various organs and tissues were surgically removed at successive time points. Briefly, after the hamsters were anesthetized with ether, skin specimens were initially collected from the areas behind the ears, and on the abdomen and back. Subsequently, peripheral organs, including the heart, lungs, liver, spleen and colon, were excised. Finally, brain samples were collected.

Preparation of tissue homogenates

Homogenates of various organs and tissues were prepared according to a previously described procedure [19]. Brain tissues were washed three times in PBS, and 10% (w/v) brain homogenates were prepared in cold lysis buffer containing a mixture of protease inhibitors (Merck, 539134, US). The buffer consisted of 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 10 mM Tris at pH 7.5. Debris was removed through low-speed centrifugation at 2,000 g for 10 min, and the supernatants were collected. For preparations of 10-fold serially diluted lysates, 10% tissue homogenates from three hamsters infected with 263K were pooled. The resulting lysates were diluted from 10^{-2} to 10^{-10} , and divided into aliquots.

Western blotting

Tissue homogenates and/or PMCA products were separated with 12% SDS-PAGE and underwent semi-dry transfer to nitrocellulose membranes. The membranes were incubated at 4°C overnight with the PrP-specific monoclonal antibody (mAb) 8H4 (1:2000 dilution, Abcam) at 37°C for 1 h, after being blocked with 5% non-fat-dried milk. After being washed four times with TBST (containing 0.1% Tween-20, pH 7.6), the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibodies (1:5000 dilution). Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) kit (PE Applied Biosystems, Foster City, CA, USA).

PMCA

PMCA was conducted in a sonicator (Misonix sonicator 3000 and 4000; Misonix, Farmingdale, NY, USA) with an adjustable temperature water bath circulation system and a microplate horn for PCR tubes. We mixed 10 µl of various diluted (10^{-1} to 10^{-8}) tissue homogenates (as the seed) with 90 µl of 10% brain homogenates of healthy hamsters (as the substrate). Thin-walled 0.2 ml PCR tubes containing 100 µl mixed sample were floated within the sonicator. One PMCA cycle consisted of a sonication step at PM 9.5 for 20 s and incubation at 37°C for 29 min and 40s. A complete direct PMCA protocol included 60 cycles for hamster-derived materials.

RT-QuIC

The details of the RT-QuIC assay were as previously described [20]. Briefly, RT-QuIC reactions contained 1 µl of various tissue homogenates from 263K-infected

hamsters, diluted from 10^{-3} to 10^{-10} , $1\times$ PBS, 170 mM NaCl, 1 mM EDTA, 0.01 mM thioflavin T (ThT), 0.001% sodium dodecyl sulfate (SDS) and 10 μ g rHaPrP90-231 in a final volume of 100 μ l. Each reaction was performed in quadruplicate. The assay was conducted in black 96-well, optical-bottomed plates (Nunc, 265301) in a BMG FLUOstar plate reader (BMG LABTECH). The working conditions were as follows: temperature, 50°C; shaking speed, 900 rpm; vibration/incubation time, 90/30 sec; and total reaction time, 90 h. ThT fluorescence (excitation wavelength, 450 nm; emission wavelength, 480 nm) was measured automatically every 30 minutes and expressed as relative fluorescence units. The cutoff value was set as the mean value of the negative controls plus three times the standard deviation. A sample was considered positive if two or more wells showed positive reaction curves.

Calculation of 50% seed dose

The SD_{50} value of each preparation was calculated according to the Spearman-Kärber method with the following formula: $\log SD_{50} = XK + C[\Pi + P(i+1)] - 1/2C$, where XK represents the logarithm of the highest dilution in which all wells were positive, C is the logarithm of the ratio of adjacent doses, and Π and $P(i+1)$ are the positivity rates of each dose group.

Ethical statement

All animal experiments were performed strictly in accordance with international and national laws, regulations and guidelines for the use and care of laboratory animals, and were approved by the National Institute for Viral Disease

Control and Prevention, Chinese Center for Disease Control and Prevention (approval no.20220221017).

RESULTS

To evaluate the RT-QuIC reactivity and the SD_{50} values of various tissues of hamsters infected with the scrapie agent 263K at various time points during incubation period, we collected brain, heart, liver, spleen, lung, colon and skin samples (from behind the ear, and from the abdomen and backside) at 20, 40, 60 or 80 days post-inoculation (dpi). After preparation of 10% tissue homogenates, lysates from three infected hamsters were pooled, and serial 10-fold dilutions of tissue homogenate (from 10^{-2} to 10^{-10}) were prepared. PrP^{res} detection in the brain tissues at the terminal stage (80 dpi) was performed separately with western blotting and PMCA. Western blot analysis of PK-digested samples with mAb 6D11 detected PrP^{res} signals at the 10^{-3} dilution (Fig 1A). Direct PMCA revealed clear PrP^{res} bands for the 10^{-5} dilution (Fig 1B). Furthermore, the 10^{-2} diluted brain homogenates from 20, 40, 60 and 80 dpi were subjected to direct PMCA. Three strong PrP^{res} bands were detected in the 80 and 60 dpi preparations, whereas relatively weak PrP^{res} bands were observed in 40 and 20 dpi preparations (Fig 1C). Similarly to the western blot findings, the diglycosylated PrP^{res} band predominated in the PMCA products.

RT-QuIC reactivity of various tissues from 263K-infected hamsters

Serial 10-fold dilutions of tissue homogenates from various organs were separately subjected to RT-QuIC assays

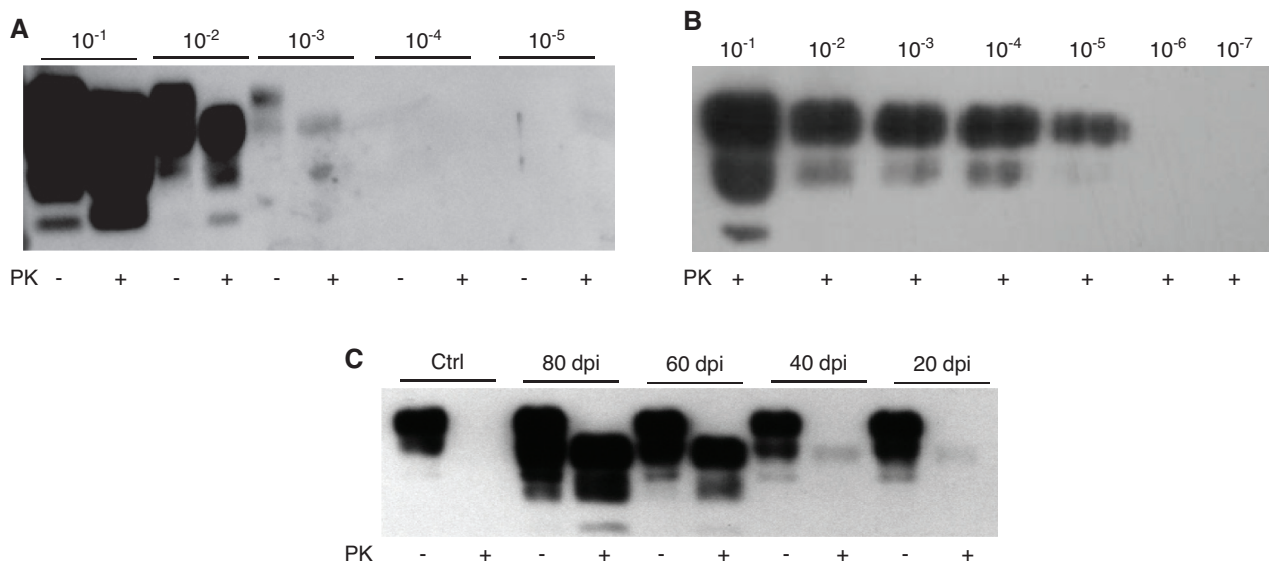


FIGURE 1 | Detection of PrP^{res} in the brain in 263K infected hamsters by western blotting and PMCA. (A) Western blot of infected brains at the terminal stage with the anti-PrP mAb 6D11. The dilutions of brain homogenates are shown above. (B) Western blot of the products of direct PMCA. Diluted brain homogenates of the infected hamsters at terminal stage were used as the seeds, and 10% brain homogenate from normal hamsters was used as the substrate. The dilutions of the input brain homogenates are shown above. (C) Western blot of the products of direct PMCA of 10% brain homogenate from infected hamsters, collected at various time points post-infection. PK+: digested with 50 mg/ml PK; PK-: without PK digestion.

TABLE 1 | RT-QuIC reactivity per well for various tissues from 263K infected hamsters, collected at four time points.

	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	Result
Brain									
20 dpi	4/4	4/4	4/4	3/4	3/4	0/0	0/0	0/0	+ in 10 ⁻⁷
40 dpi	4/4	4/4	4/4	3/4	3/4	1/4	0/0	0/0	+ in 10 ⁻⁷
60 dpi	4/4	4/4	4/4	4/4	4/4	2/4	0/0	0/0	+ in 10 ⁻⁸
80 dpi	4/4	4/4	4/4	4/4	4/4	2/4	2/4	0/0	+ in 10 ⁻⁹
Heart									
20 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
40 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
60 dpi	4/4	4/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁴
80 dpi	4/4	4/4	2/4	2/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶
Liver									
20 dpi	3/4	3/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁴
40 dpi	3/4	2/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁴
60 dpi	4/4	4/4	2/4	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	3/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
Spleen									
20 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
40 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
60 dpi	4/4	4/4	2/4	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁴
Lung									
20 dpi	1/4	1/4	0/0	0/0	0/0	0/0	0/0	0/0	-
40 dpi	3/4	3/4	1/4	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁴
60 dpi	4/4	4/4	4/4	3/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶
80 dpi	4/4	4/4	4/4	3/4	1/4	0/0	0/0	0/0	+ in 10 ⁻⁶
Colon									
20 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
40 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
60 dpi	4/4	3/4	0/0	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	3/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶
Skin (behind ear)									
20 dpi	1/4	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-
40 dpi	4/4	2/4	0/0	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
60 dpi	4/4	3/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	3/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶
Skin (abdomen)									
20 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0
40 dpi	4/4	2/4	0/0	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
60 dpi	4/4	3/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	3/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶
Skin (backside)									
20 dpi	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0
40 dpi	4/4	2/4	0/0	0/0	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
60 dpi	4/4	3/4	1/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁵
80 dpi	4/4	4/4	3/4	1/4	0/0	0/0	0/0	0/0	+ in 10 ⁻⁶

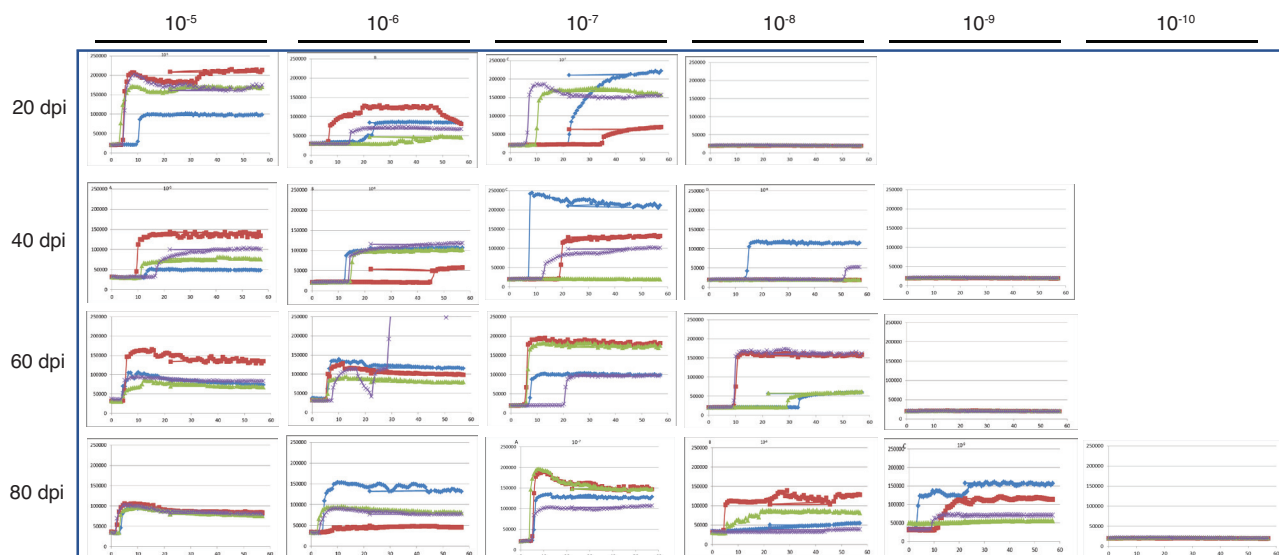


FIGURE 2 | Representative graphs of RT-QuIC of various diluted brain homogenates from 263K infected hamsters, collected at various time points post-infection. The reactive times are indicated on the X axis, and the ThT values are indicated on the Y axis. The dilutions of the brain homogenates and the sampling times post-infection are indicated above and to the right of the graphs.

with recombinant HaPrP90-231 as the substrate. The starting dilution was 10^{-3} , and each reaction was performed in quadruplicate wells. The RT-QuIC reactivity of each reaction is summarized in Table 1. For brain tissues, samples from all time points showed positive reactions in RT-QuIC, and the reactivity increased in a time-dependent manner. Specifically, the 20 and 40 dpi samples were positive at a 10^{-7} dilution, the 60 dpi samples were positive at a 10^{-9} dilution, and the 80 dpi samples were positive at a 10^{-8} dilution. In addition, the lag times showed a time-dependent decrease (Fig 2). In contrast to the high RT-QuIC reactivity and consistent positive results observed in the brain lysates, the RT-QuIC reactivity in the tested peripheral organs were markedly lower (Table 1). The heart, spleen and colon homogenates exhibited a similar reactivity profile, which was negative in 20 and 40 dpi samples; positive in 10^{-5} and 10^{-6} dilutions of 60 and 80 dpi samples from the heart and colon; and positive in 10^{-5} and 10^{-4} dilutions of 60 and 80 dpi samples from the spleen. Slightly higher RT-QuIC positivity was observed at all four time points in liver samples and the last three time points in lung samples. Positive reactions were observed in the 10^{-4} dilution of 20 and 40 dpi and the 10^{-6} dilution of 60 and 80 dpi liver samples, and in the 10^{-4} dilution of 40 dpi and the 10^{-6} dilution of 60 and 80 dpi lung samples. The reactivity of skin samples from three anatomic positions (behind the ear, and the abdomen and backside) were almost identical: positivity was observed for 10^{-5} , 10^{-5} and 10^{-6} dilutions of 40, 60 and 80 dpi samples.

RT-QuIC lag times for 60 and 80 dpi tissue samples at 10^{-4} , 10^{-5} and 10^{-6} dilutions

The lag times of the 10^{-4} , 10^{-5} and 10^{-6} diluted lysates from various tissues at 60 and 80 dpi were collected and analyzed with RT-QuIC. In Fig 3, each dot represents the

lag time for each well. Generally, the lag times and the numbers of positive wells for the tested tissues (Fig 3A) were longer and fewer, respectively, at 60 dpi than 80 dpi (Fig 3B). The lag times of both 60 and 80 dpi brain tissues were significantly shorter than those for the other peripheral tissues, particularly at 10^{-5} and 10^{-6} dilutions. The lag times at those three dilutions were slightly shorter for liver tissues than other internal organ tissues, whereas the lag times for heart and colon tissues were longer than other internal organ tissues. Moreover, 60 dpi samples showed shorter lag times, particularly at the 10^{-4} dilution, for skin samples from behind the ear than from the abdomen and backside. In the 80 dpi preparations, the lag times of the three skin specimen types were comparable.

Calculation of SD_{50} values in RT-QuIC for tissues at various time points post infection

The SD_{50} values (reported in $\log_{10} SD_{50}$ units/2 μ l) of each tested homogenate was calculated with the Spearman-Kärber method. As shown in Fig 4, the SD_{50} values for all tested tissues increased with the duration of incubation. Brain tissues had notably high SD_{50} values (7.00, 7.25, 8.00 and 8.50 in 20, 40, 60 and 80 dpi samples, respectively). The SD_{50} values of were uncalculatable (designated as 0.00) in the 20 and 40 dpi heart, liver, spleen, lung and colon samples; the values increased to the range of 4.25–6.25 in 60 dpi samples and plateaued in 80 dpi samples. The three skin sample types showed low SD_{50} values (3.50) at 40 dpi, whereas these values increased to 4.5–4.75 at 60 dpi and to 5.50 at 80 dpi. These data indicated that the prion seeds capable of eliciting positive RT-QuIC reactions were widely distributed in CNS tissues and peripheral organs of the prion infected experimental hamsters. The reactive capacity of prion seeds increased with incubation time after prion infection, and

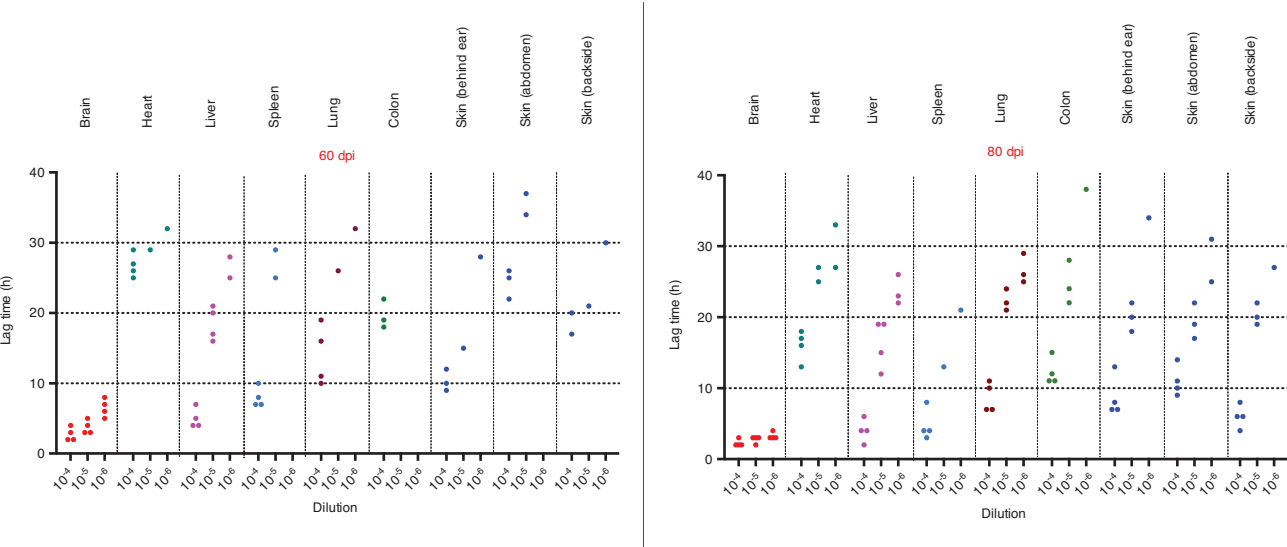


FIGURE 3 | Lag times of 10^{-3} , 10^{-4} and 10^{-5} diluted tissue lysates from 263K infected hamsters collected at 60 (left) and 80 (right) dpi. The dots represent wells showing positive reactivity. The dilutions of the tissue lysates are indicated on the X-axis, and the lag times (h) are indicated on the Y-axis. The tissue specimens are illustrated above.

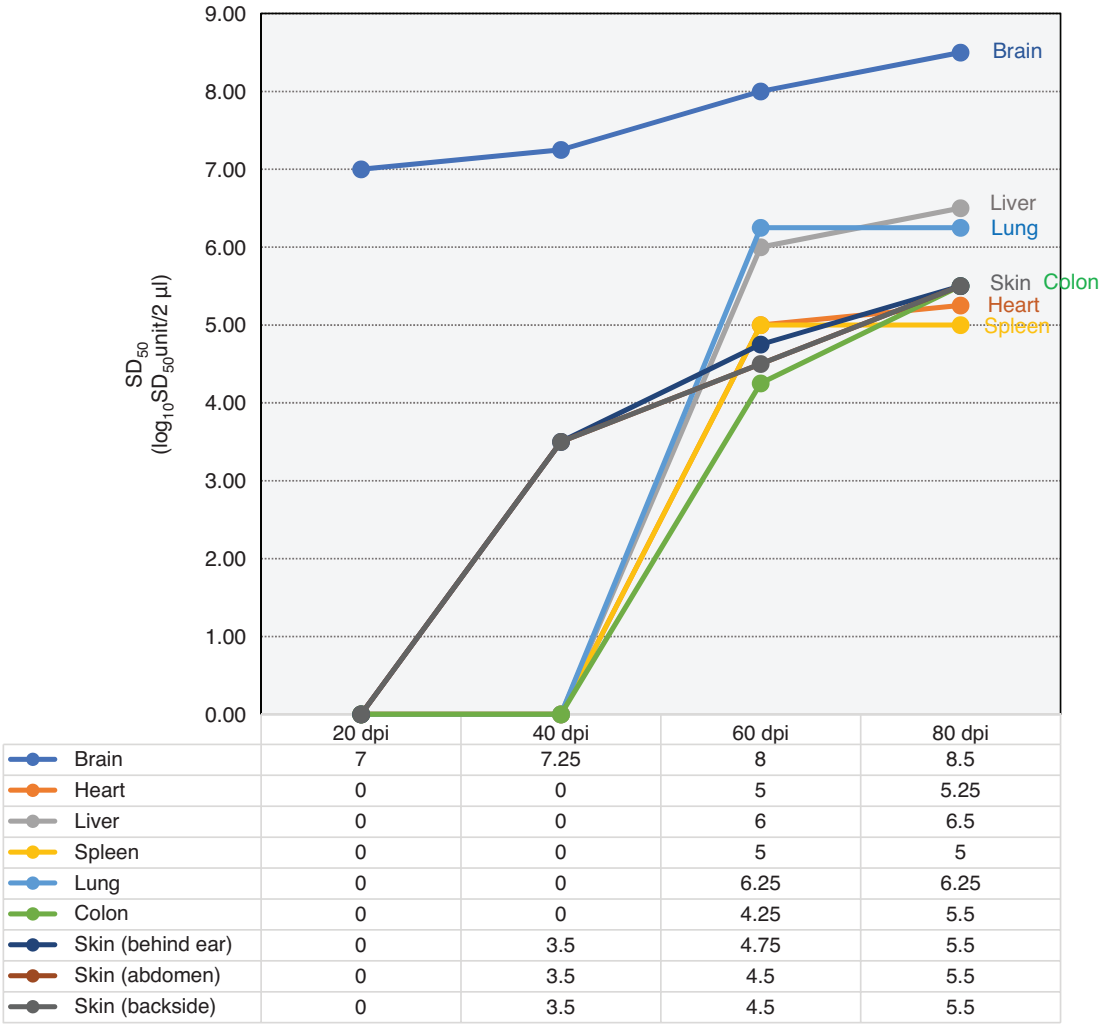


FIGURE 4 | Calculated RT-QulC SD_{50} values for various organs and tissues from 263K infected hamsters, collected at 20, 40, 60 and 80 dpi. Y-axis: SD_{50} values ($\log_{10} SD_{50}$ units/ $2 \mu l$). The exact SD_{50} data for various organs and tissues are shown at the bottom.

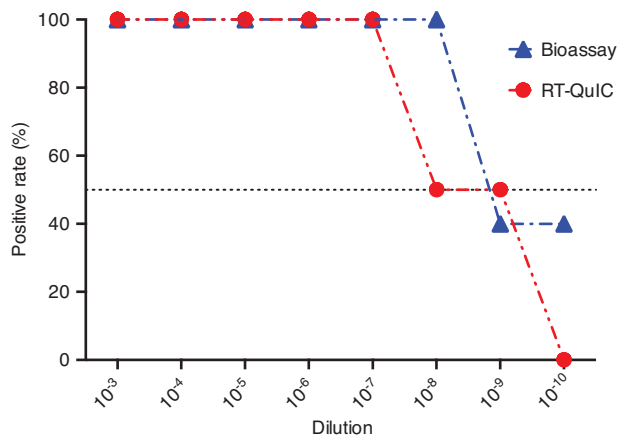


FIGURE 5 | Comparison of the positivity rates (%) in RT-QuIC and bioassays of various diluted brain homogenates from 263K infected hamsters. The RT-QuIC positivity rate is the percentage of wells showing positive RT-QuIC reactions per dilution. The bioassay positivity rate is the percentage of intracerebrally inoculated hamsters showing fatal outcomes per dilution. X-axis: brain homogenate dilutions; Y-axis: positivity rate (%).

the titer of prion seeds in the CNS tissues was significantly higher than that in peripheral tissues.

Comparison of positivity rates in RT-QuIC and fatal rates with bioassays

The 50% lethal dose (LD_{50}) for intracerebral inoculation of the scrapie agent 263K in hamsters has been reported to be $9.2 \log_{10} LD_{50}$ i.c. units/0.001 g brain, according to the Reed-Muench method [21]. To examine the correlation between the SD_{50} in RT-QuIC and LD_{50} in bioassays, we separately calculated the RT-QuIC positivity rates of reactive wells per dilution for brain tissues at 80 dpi and the fatality rates of intracerebrally infected hamsters per dilution. In dilutions from 10^{-3} to 10^{-7} , all wells in the RT-QuIC were positive, and all inoculated animals died. The RT-QuIC positivity rate decreased to 50% for the 10^{-8} and 10^{-9} dilutions, and all wells were negative for the 10^{-10} dilution, whereas the fatal rates of the infected hamsters were 100% for the 10^{-8} dilution but decreased to 40% for both the 10^{-9} and 10^{-10} dilutions (Fig 5). These findings highlighted a strong correlation between RT-QuIC positivity rates and fatality rates in the bioassays in 263K-infected hamsters, across a wide range of dilutions.

DISCUSSION

Herein, we systematically assessed the RT-QuIC seeding capacity of various organs and tissues from hamsters infected with the 263K agent at various time points after inoculation. The investigated organ and skin areas were selected for their importance and representativeness. Positive RT-QuIC reactions were elicited from all tested tissues at the terminal stage (80 dpi) and middle-late stage (60 dpi) of infection, thereby highlighting a broad distribution of prion seeds in both the CNS and peripheral

tissues of scrapie infected experimental hamsters. Notably, the RT-QuIC seeding capacity of the infected brains was much stronger than observed for peripheral tissues; we observed positivity in early stage (20 dpi) samples and in reactions using highly diluted specimens. Skin and lung tissues were identified as the second and third most infectious areas, respectively.

Prions in the brain and other neuropathological abnormalities can be detected long before the clinical manifestations of prion diseases present [22]. Although several methods have been described to detect prion signals or other biomarkers of prion diseases in the urine and serum [23–26], clinically applicable peripheral tissue-based tools were unavailable before the development of skin RT-QuIC. Recently, skin RT-QuIC has been widely used in the diagnosis of human prion diseases, particularly sporadic CJD [27–29]. Three skin specimen types from various anatomic positions of 263K infected hamsters showed similar RT-QuIC reactivity. Importantly, positive skin RT-QuIC was detectable in the early middle stage (40 dpi) samples. Although our data from scrapie infected hamsters cannot be directly extended to human prion disease, the sensitive RT-QuIC reactivity in skin specimens might provide useful scientific clues for the application of skin RT-QuIC before the onset of human prion diseases. Additionally, the wide distribution of prion seeds for RT-QuIC in the tissues of internal organs at late and terminal stage provide additional options for sampling. In fact, our early studies have described the prion seeding capacity of the spleen and muscle in 263K infected hamsters in PMCA [30,31]. This study, building on our previous research findings, used RT-QuIC to determine the potential transmission risk and detection capacity of prion diseases across various tissues. These results provide valuable insights and possible directions for further exploration of prion occurrence and deposition in other bodily sites, thereby contributing to understanding of prion disease progression and dissemination within the body.

Prion diseases pose zoonotic risks, because of their ability to spread between humans and animals. Therefore, investigating prion distribution and RT-QuIC reactivity in the hamster model offers insights into potential prion distribution in humans and other animals, and provides a scientific foundation for addressing related public health issues and ultimately decreasing associated public health risks. We determined the RT-QuIC SD_{50} values for various tissues collected from 263K intracerebrally infected hamsters at various times. Although some tissues in middle-early stage showed RT-QuIC positivity at the lowest dilution (10^{-3}), their SD_{50} values were uncalculatable with the Spearman-Kärber method, because not all wells showed positivity at the lowest preparation dilutions. The RT-QuIC SD_{50} values of brain tissues ranged from 7.00 at the early stage to 8.50 at the final stage, and these values were significantly higher than observed for peripheral tissues. Our previous study of intracerebral 263K challenge in hamsters determined an LD_{50} value of $9.2 \log_{10} LD_{50}$ i.c. units/0.001 g

brain with the Reed-Muench method [21]; therefore, 1 LD₅₀ unit contains approximately 7.5 log₁₀ LD₅₀ i.e. units/2 µl. Those two values are not directly comparable: LD₅₀ represents the lethality of prions after intracerebral inoculation *in vivo*, whereas SD₅₀ is the seeding capacity of prions for fibrillation in RT-QuIC *in vitro*. Unlike the products of PMCA, the infectivity of RT-QuIC products remains questionable. However, the strong correlation of positivity rates in serial dilutions of brain homogenates of 263K infected hamsters between the RT-QuIC and bioassay indicated the possibility of using the RT-QuIC SD₅₀ instead of LD₅₀ for at least partially determining the risk of biomaterials from prion infected individuals.

Because RT-QuIC is an ultrasensitive assay, the potential for contamination is a concern, given that contamination is frequently observed in routine PCR. To avoid this potential problem, we used several quality control measures. During the sampling process, we collected skin specimens first, then the internal organs and finally the brains, ensuring that the material containing the highest prion dose was collected last. During the sample addition process, we started with highly diluted preparations, then moved to lower dilutions. Each assay included a negative control (brain homogenates from a healthy hamster) as well as control reagents. Moreover, automatic detection of ThT values without uncapping the reaction efficiently avoided the contamination of RT-QuIC products. The clear dose-dependent RT-QuIC reactivity, indicated by gradually decreasing positivity rates and increasing lag times with dilution, demonstrated the minimal influence of contamination in the RT-QuIC in this study.

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CONFLICTS OF INTEREST

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REFERENCES

1. Prusiner SB. Prions. *Proc Natl Acad Sci U S A*. 1998;95(23):13363-13383.
2. Chen C, Dong XP. Epidemiological characteristics of human prion diseases. *Infect Dis Poverty*. 2016;5(1):47.
3. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature*. 2001;411(6839):810-813.
4. Peden AH, McGuire LI, Appleford NEJ, Mallinson G, Wilham JM, Orrú CD, et al. Sensitive and specific detection of sporadic Creutzfeldt-Jakob disease brain prion protein using real-time quaking-induced conversion. *J Gen Virol*. 2012;93(Pt 2):438-449.
5. Xiao K, Yang XH, Zou WQ, Dong XP, Shi Q. Assessment of the sensitivity and specificity of the established real-time quaking-induced conversion (RT-QuIC) technique in Chinese CJD surveillance. *Biomed Environ Sci*. 2020;33(8):620-622.
6. Schmitz M, Villar-Piqué A, Hermann P, Escaramís G, Calero M, Chen C, et al. Diagnostic accuracy of cerebrospinal fluid biomarkers in genetic prion diseases. *Brain*. 2022;145(2):700-712.
7. Orrú CD, Yuan J, Appleby BS, Li B, Li Y, Winner D, et al. Prion seeding activity and infectivity in skin samples from patients with sporadic Creutzfeldt-Jakob disease. *Sci Transl Med*. 2017;9(417):eaam7785.
8. Ramasamy I, Law M, Collins S, Brooke F. Organ distribution of prion proteins in variant Creutzfeldt-Jakob disease. *Lancet Infect Dis*. 2003;3(4):214-222.
9. Kumagai S, Daikai T, Onodera T. Bovine spongiform encephalopathy – a review from the perspective of food safety. *Food Saf (Tokyo)*. 2019;7(2):21-47.
10. Davidson LRR. *Diagnostic Utility of RT-QuIC for the Diagnosis of Sporadic Creutzfeldt-Jakob disease*. Scotland: Edinburgh Research Archive, University of Edinburgh; 2019.
11. Douet JY, Huor A, Cassard H, Lugan S, Aron N, Arnold M, et al. Wide distribution of prion infectivity in the peripheral tissues of vCJD and sCJD patients. *Acta Neuropathol*. 2021;141(3):383-397.
12. Turner ML. Safety of blood, blood derivatives, and plasma-derived products. *Handb Clinical Neurol*. 2018;153:463-472.
13. Pauli G. Tissue safety in view of CJD and variant CJD. *Cell Tissue Bank*. 2005;6(3):191-200.
14. López FJG, Ruiz-Tovar M, Almazán-Isla J, Alcalde-Cabero E, Calero M, de Pedro-Cuesta J. Risk of transmission of sporadic Creutzfeldt-Jakob disease by surgical procedures: systematic reviews and quality of evidence. *Euro Surveill*. 2017;22(43):16-00806.
15. Henderson DM, Denkers ND, Hoover CE, Garbino N, Mathiason CK, Hoover EA. Longitudinal detection of prion shedding in saliva and urine by chronic wasting disease-infected deer by real-time quaking-induced conversion. *J Virol*. 2015;89(18):9338-9347.
16. Wilham JM, Orrú CD, Bessen RA, Atarashi R, Sano K, Race B, et al. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. *PLoS Pathog*. 2010;6(12):e1001217.
17. Xiao K, Shi Q, Zhou W, Gao C, Chen C, Zhang BY, et al. Evaluation of the effect of various main elements on the PrPsc detection by real-time quaking-induced conversion assay. *Int J Mol Med*. 2018;42(6):3231-3237.
18. Xiao K, Zhang BY, Zhang XM, Wang J, Chen C, Chen LN, et al. Re-infection of the prion from the scrapie-infected cell line SMB-S15 in three strains of mice, CD1, C57BL/6 and Balb/c. *Int J Mol Med*. 2016;37(3):716-726.
19. Shi Q, Zhang BY, Gao C, Zhang J, Jiang HY, Chen C, et al. Mouse-adapted scrapie strains 139A and ME7 overcome species barrier to induce experimental scrapie in hamsters and changed their pathogenic features. *Virol J*. 2012;9:63.
20. Xiao K, Shi Q, Zhou W, Zhang BY, Wang Y, Chen C, et al. T188K-familial Creutzfeldt-Jacob disease, predominant among Chinese, has a reactive pattern in CSF RT-QuIC different from D178N-fatal familial insomnia and E200K-familial CJD. *Neurosci Bull*. 2019;35(3):519-521.
21. Shi Q, Zhang BY, Gao C, Han J, Zhou YL, Dong XP. Measurement of lethal dose 50 (LD₅₀) of scrapie strain 263K in hamsters through intracerebral route. *Chin J Zoonoses*. 2006;22:252-255.
22. Soto C, Anderes L, Suardi S, Cardone F, Castilla J, Frossard MJ, et al. Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. *FEBS Lett*. 2005;579(3):638-642.
23. Zeiler B, Adler V, Kryukov V, Grossman A. Concentration and removal of prion proteins from biological solutions. *Biotechnol Appl Biochem*. 2003;37(Pt 2):173-182.
24. Freudenberg JA, Bembas K, Greene MI, Zhang H. Non-invasive, ultra-sensitive, high-throughput assays to quantify rare biomarkers in the blood. *Methods*. 2008;46(1):33-38.

25. Andrievskaia O, Algire J, Balachandran A, Nielsen K. Prion protein in sheep urine. *J Vet Diagn Invest.* 2008;20(2):141-146.
26. Reichl H, Balen A, Jansen CA. Prion transmission in blood and urine: what are the implications for recombinant and urinary-derived gonadotrophins? *Hum Reprod.* 2002;17(10):2501-2508.
27. Xiao K, Yang X, Zhou W, Chen C, Shi Q, Dong X. Validation and application of skin RT-QuIC to patients in China with probable CJD. *Pathogens.* 2021;10(12):1642.
28. Mammana A, Baiardi S, Rossi M, Franceschini A, Donadio V, Capellari S, et al. Detection of prions in skin punch biopsies of Creutzfeldt-Jakob disease patients. *Ann Clin Transl Neurol.* 2020;7(4):559-564.
29. Wang Z, Manca M, Foutz A, Camacho MV, Raymond GJ, Race B, et al. Early preclinical detection of prions in the skin of prion-infected animals. *Nat Commun.* 2019;10(1):247.
30. Shi S, Dong CF, Wang GR, Wang X, An R, Chen JM, et al. PrP(Sc) of scrapie 263K propagates efficiently in spleen and muscle tissues with protein misfolding cyclic amplification. *Virus Res.* 2009;141(1):26-33.
31. Gao C, Han J, Zhang J, Wei J, Zhang BY, Tian C, et al. Protein misfolding cyclic amplification cross-species products of mouse-adapted scrapie strain 139A and hamster-adapted scrapie strain 263K with brain and muscle tissues of opposite animals generate infectious prions. *Mol Neurobiol.* 2017;54(5):3771-3782.