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Monitoring of chronic wasting disease using real-time quaking-induced conversion assay in Japan

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ABSTRACT. There has been no report on Chronic wasting disease (CWD) cases in Japan to date; however, there is concern about the geographic spread of CWD. To clarify the CWD status in Japan, we conducted CWD monitoring using real-time quaking-induced conversion (RT-QuIC) assay which can detect the low level of CWD prions. A total of 690 obex samples collected from sika deer and Reeves's muntjac in Hokkaido and Honshu was tested for CWD prions. No CWD-positive cases were found, suggesting that CWD is nonexistent in Japan. Our results also indicate that RT-QuIC assay is useful for continuous monitoring of CWD. Furthermore, nucleotide sequence analysis of the PrP gene revealed sika deer in Japan harbor CWD susceptible allele.

KEY WORDS: chronic wasting disease, prion, prion protein, real-time quaking-induced conversion, scrapie

Chronic wasting disease (CWD) is a prion disease in the wild cervid, which is progressive and ultimately fatal. Since CWD was first identified in a group of captive mule deer in 1967, CWD cases in cervids, such as white-tailed deer, mule deer, elk, and reindeer have been disclosed in the USA, Canada, South Korea [5], and recently in Scandinavian countries [12, 21]. Unlike other prion diseases, CWD prions, the causative agents of CWD, are secreted from infected animals into body fluids and excretions such as saliva, urine, and feces [14]. Compared with bovine spongiform encephalopathy (BSE), CWD transmission does not occur in mice expressing human PrP [11]; however, the zoonotic potential of CWD cannot be ignored as CWD prions can be transmitted experimentally to several animals including the squirrel monkey [18].

Recently, venison consumption in Japan is getting popular yearly. From 2017 to 2019, the amount of processed sika deer (*Cervus Nippon*) meat at the slaughterhouses increases by nearly 150 tons every year in Japan [16]. Therefore, clarifying the CWD status in Japan is important for ensuring the safety and security of deer products. There are two reports on CWD surveillance in Japan that were conducted more than 15 years ago, and no CWD cases were identified at that time. [10, 13]. However, the recent emergence and spread of CWD in Scandinavian countries remind us the importance of continuous monitoring to clarify the CWD status. In this study, we attempted to clarify a recent CWD status in Japan using real-time quaking-induced conversion (RT-QuIC) assay.

The RT-QuIC assay is a specific and highly sensitive method used in detecting the amyloid seeding activity of an abnormal isoform of the prion protein (PrP^{Sc}) [3]. PrP^{Sc} is a major component of prions, and the presence of PrP^{Sc} indicates the existence of prions. The assay can detect low levels of PrP^{Sc} from tissues and body fluids of prion-infected animals within a short time. Despite its specificity and sensitivity, the reaction easily interferes with inhibitory factors present in the tissue homogenates or body fluids, that impedes the detection of PrP^{Sc} from the high concentration of tissue homogenates. We recently demonstrated that the recombinant cervid PrP (rCerPrP) was a suitable substrate for detecting low levels of CWD and atypical BSE prions in a high concentration of brain tissue homogenates [20]. Since the reaction of rCerPrP is less affected by the tissue homogenates present in

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the reaction mixture than other rPrPs [20], RT-QuIC assay using rCerPrP will be useful as a practical and reliable method for the monitoring of CWD.

A total of 690 obex regions of the medulla oblongata from deer captured or hunted in Japan during 2016–2020 were tested in this study (Fig. 1 and Table 1). The 634 obex tissues were obtained from three and one game meat processing companies in Hokkaido and Hyogo prefecture, respectively, and 56 obex tissues of Reeves's muntjac in Kanto area were kindly provided by Tokyo metropolitan Oshima Park Office. The tested deer included 332 males and 275 females. Gender information was unavailable for the remaining 83 samples; the estimated age ranged from 0 to 8 year-old, while the age of 70 samples was unknown (Table 1). The obex tissues (100 mg) were homogenized in sterile phosphate-buffered saline (PBS) at a concentration of 10% and stored at −30°C until use. Prior to the first RT-QuIC assay, the brain homogenates (BH) except for those from Tomuraushi were pretreated using a fully automatic cross-ultrasonic protein activating apparatus Elestein 070-GOT (Elekon Science, Chiba, Japan) with 10 cycles of intermittent sonication and agitation in the cold water. One cycle was comprised of 6 repetitions of 30 sec pulse on and 10 sec pulse off followed by 2 min agitation. The BH samples from Tomuraushi were pretreated solely by the sonication for 2 min at 35% amplitude using an Ultrasonic Cup-horn Digital Sonifier (Branson, Danbury, CT, USA). Then, 2% BH was prepared with PBS.

The RT-QuIC assay was performed as described elsewhere [20], using infinite F200 and M200 microplate readers (TECAN, Männedorf, Switzerland). The duration of the RT-QuIC assay was set for a maximum of 40 hr. CWD-positive and negative BHs were kindly provided by Drs. Edward A Hoover, Candace K Mathiason, and Nathaniel D Denkers, Colorado State University, USA, and have been used as seeds in the previous study [20]. Two-percent BH of pooled six CWD-affected deer from the USA

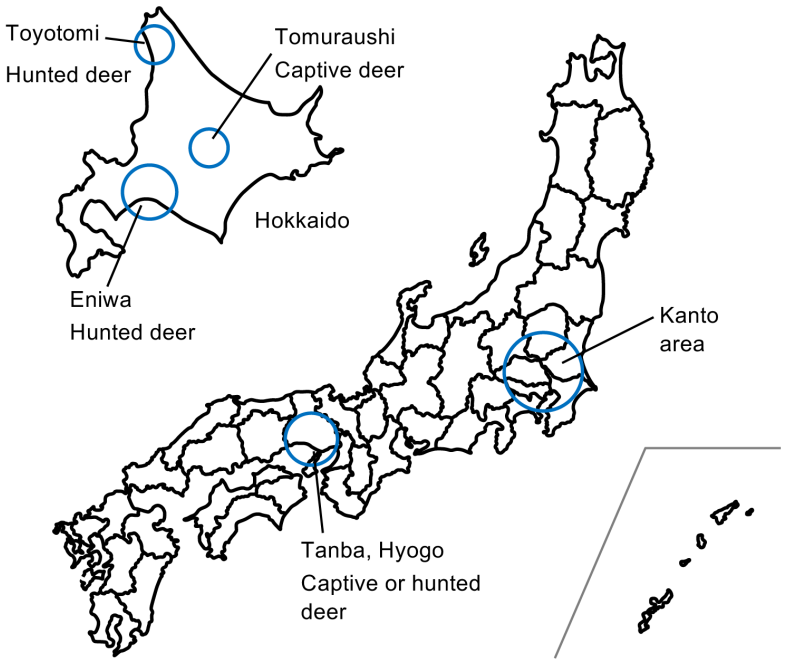


Fig. 1. Areas of sampling. The areas where deer were captured or hunted were shown in blue circles on the map. Names of area, and prefecture, and capture methods were shown around the circles. The capture area in Kanto area is still undisclosed. Detailed information were provided in Table 1.

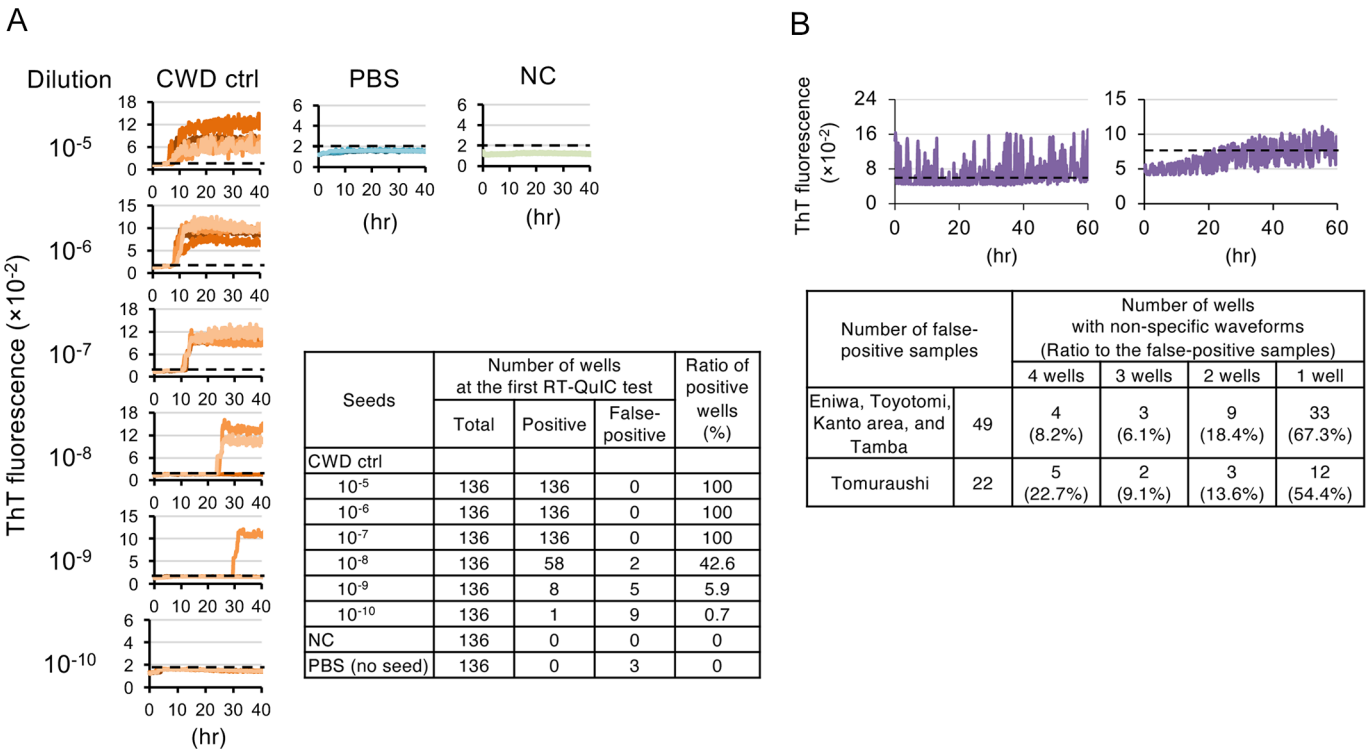
Table 1. Summary of deer obex samples

Capture areas	Number	Sex			Age									
		Male	Female	Unknown	0	1	2	3	4	5	6	7	8	Unknown
Hokkaido														
Eniwa	340	186	154	0	8	35	80	80	60	38	31	5	1	2
Tomuraushi	53	9	21	23	0	0	4	17	21	0	5	0	0	6
Toyotomi	61	41	19	1	0	0	27	17	14	1	1	0	0	1
Kanto area [a)]	56			56										56
Hyogo														
Tanba	180	96	81	3	0	4	21	62	52	21	12	3	0	5
Total	690	332	275	83	8	39	132	176	147	60	49	8	1	70

a) Sex and age of Reeves's muntjac in Kanto area were not applicable.

was serially diluted 10-fold with PBS from 0.02% to 0.0000002% and 5 µl of each dilution was used as seeds for the positive control (final dilution in the reaction mixture: 10^{-5}–10^{-10}, corresponding to 0.001% to 0.00000001%) (hereafter referred to as CWD ctrl). The 2% BH of pooled two CWD-unaffected white-tailed deer in the USA was used as the negative control (NC). The first RT-QuIC tests were conducted with quadruplicate wells. The sample was evaluated as positive if thioflavin T (ThT) intensity exceeded the threshold calculated as mean ThT intensity plus 5 ×SD from the wells without seed (PBS). The lag phase (h) was defined as the time of reaction needed to cross the threshold [7, 20]. If the ThT intensity exceeded the threshold, but the ThT fluorescence curve showed atypical forms in more than one out of four wells, such a sample was considered false-positive and was subjected to re-examination in the next RT-QuIC assay with an increased number of wells. For nucleotide sequence determination of PrP gene, genomic DNA was isolated from 25 mg brain tissues or 100 µl of 10% BHs using DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). The gene fragment including the *PRNP* was amplified using primers, MD582F and MD1479R described elsewhere [9], and the nucleotide sequences were determined using four primers; MD582F, MD1479R, DPrPF1: 5′-TGGCTACATGCTGGGAAGTG-3′, and DPrPR1: 5′-TTTTCACGATAGTAACGGTC-3′.

The first RT-QuIC tests were performed with quadruplicate wells of PBS, NC, CWD ctrl (10^{-5}–10^{-10}), and samples examined, which were arranged in the same plate. A total of 136 wells for each control was used at the first RT-QuIC tests. Figure 2A shows the representative ThT fluorescence curves from one RT-QuIC assay and the summarized results of RT-QuIC assay of the first RT-QuIC tests. Although typical prolongation of lag phases was observed with the dilution of positive controls for CWD, reactions were positive up to 10^{-9} dilution in the representative RT-QuIC assay. In a total first RT-QuIC tests for monitoring, typical ThT fluorescence curves that exceeded the threshold were observed in 100% wells at 10^{-5}–10^{-7}, 42.6% wells at 10^{-8}, 5.9% wells at 10^{-9}, and 0.7% at 10^{-10} CWD ctrl dilutions. No positive or false-positive reaction was observed in the wells of NC (with uninfected deer brain homogenates) throughout the examinations. Atypical ThT fluorescence curves such as oscillated waveforms and gradual elevation of the base line were occasionally observed (representative atypical ThT fluorescence curves are indicated in Fig. 2B). These curves exceeded the threshold but were clearly distinguished from the ThT fluorescence curves of CWD ctrl (Fig. 2A) based



Seeds	Number of wells at the first RT-QuIC test			Ratio of positive wells (%)
	Total	Positive	False-positive	
CWD ctrl				
10^{-5}	136	136	0	100
10^{-6}	136	136	0	100
10^{-7}	136	136	0	100
10^{-8}	136	58	2	42.6
10^{-9}	136	8	5	5.9
10^{-10}	136	1	9	0.7
NC	136	0	0	0
PBS (no seed)	136	0	3	0

Number of false-positive samples		Number of wells with non-specific waveforms (Ratio to the false-positive samples)			
		4 wells	3 wells	2 wells	1 well
Eniwa, Toyotomi, Kanto area, and Tamba	49	4 (8.2%)	3 (6.1%)	9 (18.4%)	33 (67.3%)
Tomuraushi	22	5 (22.7%)	2 (9.1%)	3 (13.6%)	12 (54.4%)

Fig. 2. Representative thioflavin T fluorescence curves from chronic wasting disease positive control and false-positive samples. **(A)** Representative fluorescence curves of chronic wasting disease (CWD)-positive and negative control from one real-time quaking-induced conversion (RT-QuIC) assay (left) and summarized results at the first RT-QuIC tests (right). Brain homogenates of CWD-infected and uninfected deer (CWD ctrl (orange) and NC (light green), respectively) were used as positive and negative controls. The quadruplicate wells without seed (PBS (light blue)) were used to calculate the threshold (dashed lines). Data shown here were collected using infinite M200 microplate reader. The numbers of the total wells used in the first RT-QuIC tests, and positive and false-positive wells are summarized in the table. **(B)** Summary of false-positive samples at the first RT-QuIC tests. Representative atypical thioflavin T fluorescence curves (purple), oscillated waveform (left), and gradually elevated baseline (right) are shown. Dashed lines indicate the threshold level. Data shown here were collected using infinite F200 microplate reader, which gave 3.6 times higher base line than that of M200 used in the present study. The numbers of false-positive samples, number of wells that showed non-specific waveforms among quadruplicate wells, and the percentage of false-positive samples which showed non-specific waveforms in 1 to 4 wells, are summarized in the table.

Table 2. Summary of the real-time quaking-induced conversion tests for chronic wasting disease monitoring

Capture areas (number of samples)	Rounds of RT-QuIC test [a)]					
	First		Second		Third	
	Negative	False-positive	Negative	False-positive	Negative	False-positive
BH samples pretreated with intermittent sonication and agitation						
Hokkaido						
Eniwa (340)	320 (94.1%)	20 (5.9%)	18 (5.3%)	2 (0.6%)	2 (0.6%)	0 (0%)
Toyotomi (61)	55 (90.2%)	6 (9.8%)	6 (9.8%)	0 (0%)	0 (0%)	0 (0%)
Kanto area (56)	52 (92.9%)	4 (7.1%)	4 (7.1%)	0 (0%)	0 (0%)	0 (0%)
Hyogo						
Tanba (180)	161 (89.4%)	19 (10.6%)	18 (10.0%)	1 (0.6%)	1 (0.6%)	0 (0%)
Total (637)	588 (92.3%)	49 (7.7%)	46 (7.2%)	3 (0.5%)	3 (0.5%)	0 (0%)
BH samples pretreated with a regular cup-horn sonicator						
Tomuraushi	31 (58.5%)	22 (41.5%)	19 (35.8%)	3 (5.7%)	3 (5.7%)	0 (0%)
Sum total (690)	619 (89.7%)	71 (10.3%)	65 (9.6%)	6 (0.9%)	6 (0.9%)	0 (0%)

a) The values in parentheses indicate the percentage of samples judged as negative or false-positive relative to the total number of samples from each district or total numbers of corresponding samples. RT-QuIC: real-time quaking induced conversion, BH: brain homogenate.

on a lack of significant increase of the ThT fluorescence and their appearance at a very early time point. False-positive reactions were observed in 2, 5, and 9 wells at 10^{-8}, 10^{-9}, 10^{-10} dilution of CWD ctrl, and 3 wells for no seed control (PBS) (Fig. 2A). In the first RT-QuIC tests, the atypical fluorescence curves were observed from 71 out of 690 samples (10.3%) and those samples were judged as false-positive (Table 2). During the first RT-QuIC tests, more than half of the false-positive samples showed atypical curves only in one of four wells (Fig. 2B). Compared to the incidence of atypical fluorescence curves in the samples from Tomuraushi that were pretreated with sonication using a regular cup-horn sonicator (41.5%, Table 2), the incidence of the atypical fluorescence curves apparently decreased in samples pretreated with the intermittent 10 cycles of sonication and agitation (7.7%). These results indicate that the intermittent sonication and agitation performed prior to the assays could efficiently reduce the appearance of atypical fluorescence curves if not all. A total of 71 samples with atypical fluorescence curves were subjected to the second RT-QuIC tests with octuplicate wells to confirm the results. Of 71 samples, the atypical fluorescence curves were observed in 6 samples (0.9%) (Table 2). These samples were confirmed in the third RT-QuIC tests with twelve replicated wells, and all were judged as negative (Table 2).

It has been reported that amino acid polymorphisms of cervid PrP influence susceptibility to CWD; in white-tailed deer, mule deer, and elk, polymorphisms at codon 96 (G/S), 225 (S/F), and 132 (M/L), respectively, influence CWD susceptibility; codons S96 of white-tailed deer, F225 of mule deer, and L132 reduce the susceptibility to CWD [2]. Thus, we estimated the deduced amino acid sequences of PrP in deer used in this study. A total of 50 sika deer samples, 10 from Tomuraushi, 10 from Eniwa, 20 from Toyotomi, and 10 from Tanba, were used to isolate genomic DNA. Nucleotide sequences of the PrP gene of sika deer tested in this study were identical to that of the Chinese domestic sika deer reported previously [15]; all deer tested were homozygous for $Q_{96}M_{132}S_{225}$ allele, which is thought to be a CWD-susceptible allele. Although further analyses for wider regions in Japan will be necessary; these results suggest that sika deer inhabiting Japan comprise CWD-susceptible populations.

RT-QuIC assay is an easy and sensitive method for detecting PrP^{Sc} and its utility in the diagnosis of human prion diseases has been demonstrated [3]. RT-QuIC assay using N-terminal truncated recombinant hamster PrP (rHaPrP) as a substrate is useful for detecting CWD prions from lymphoid tissues [6], brain tissue, saliva, and urine [7]. In this study, we used full-length rCerPrP instead of rHaPrP, which can detect the low levels of CWD prions in the presence of 0.1% BH [20]. Samples considered false-positive due to the atypical fluorescence curves are easily expected to be negative for PrP^{Sc}. Indeed, such samples turned out negative after the following RT-QuIC tests with increased multiplicate wells (Table 2). However, RT-QuIC assay is sensitive enough to detect PrP^{Sc} below the detection limit of western blotting (WB) and ELISA, a gold standard for PrP^{Sc} detection. Therefore, if samples show typical fluorescence curves with the long lag phase that suggests very low PrP^{Sc} level, it is difficult to confirm the result with WB and ELISA. Protein misfolding cyclic amplification (PMCA) is the sole candidate that validates such samples for the presence of prions, since a sequential PMCA is also highly sensitive for detecting the low levels of PrP^{Sc} [19].

Since atypical BSE cases have been mainly identified in cattle over eight-year-old, atypical BSE is hypothesized to be a sporadic disease in aged cattle, as is sporadic Creutzfeldt-Jakob disease in humans [8]. As the prion diseases in ruminant, the possibility of CWD occurring naturally in aged deer cannot be ruled out. Additionally, Japan imports large amounts of dry pasture and hay cube from the U.S. and Canada every year [1]. CWD prions from carcasses of CWD-affected deer that died outside and/or excreted into the environment can contaminate pastures and soil, and they persist for many years once excreted into the environment [5, 17]. Therefore, even if no live deer are imported, if hay production areas overlap with CWD outbreak areas, there is a possibility that CWD prions will enter Japan with the imported hay. In this study, CWD status was monitored about 10 years after the previous report [13]. No CWD cases were again disclosed in Japan. CWD spreads horizontally from deer to deer, whichever in the field or

in the ranch [4]. Additionally, there is an increase in demands on venison in Japan [16]. Thus, continuous monitoring is necessary for an earlier response and securing the safety of deer products.

CONFLICT OF INTEREST. The authors do not have any conflicts of interest to declare.

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