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COMPARISON OF CHRONIC WASTING DISEASE DETECTION METHODS AND PROCEDURES: IMPLICATIONS FOR FREE-RANGING WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) SURVEILLANCE AND MANAGEMENT

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ABSTRACT: Throughout North America, chronic wasting disease (CWD) has emerged as perhaps the greatest threat to wild cervid populations, including white-tailed deer (WTD; *Odocoileus virginianus*). White-tailed deer are the most sought-after big game species across North America with populations of various subspecies in nearly all Canadian provinces, the contiguous US, and Mexico. Documented CWD cases have dramatically increased across the WTD range since the mid-1990s, including in Minnesota, US. CWD surveillance in free-ranging WTD and other cervid populations mainly depends upon immunodetection methods such as immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) on medial retropharyngeal lymph nodes and obex. More recent technologies centered on prion protein amplification methods of detection have shown promise as more sensitive and rapid CWD diagnostic tools. Here, we used blinded samples to test the efficacy of real-time quaking-induced conversion (RT-QuIC) in comparison to ELISA for screening tissues collected in 2019 from WTD in southeastern Minnesota, where CWD has been routinely detected since 2016. Our results support previous findings that RT-QuIC is a more sensitive tool for CWD detection than current antibody-based methods. Additionally, a CWD testing protocol that includes multiple lymphoid tissues (e.g., medial retropharyngeal lymph node, parotid lymph node, and palatine tonsil) per animal can effectively identify a greater number of CWD detections in a WTD population than a single sample type (e.g., medial retropharyngeal lymph nodes). These results show that the variability of CWD pathogenesis, sampling protocol, and testing platform must be considered for the effective detection and management of CWD throughout North America.

Key words: CWD, diagnostics, ELISA, IHC, RT-QuIC, sampling, testing protocol.

INTRODUCTION

Chronic wasting disease (CWD) is a contagious, 100% fatal neurodegenerative disease affecting cervids. Classified as a transmissible spongiform encephalopathy, CWD is caused by a misfolded prion protein (PrP^{CWD}) which is shed through bodily fluids and can remain infectious in the environment for years (Williams and Young 1980; Prusiner 1982). Originally detected in Colorado mule deer (*Odocoileus hemionus*) in 1967, CWD has been detected in additional cervid species and expanded in geographic distribution (Williams and Miller 2002). As of June 2021, CWD has been found in at least 26 states in the US and three Canadian provinces. The continued expansion of CWD across North America, and recent detections in Scandinavian countries (Mysterud et al. 2020) is changing how cervids are hunted, managed, and consumed. For these reasons, stakeholders tasked with managing CWD must have access to the best diagnostic tools and relevant protocols.

The Minnesota Department of Natural Resources (MNDNR) has surveyed free-ranging white-tailed deer (*Odocoileus virgin-*

ianus) for CWD since detection in 2002 in farmed elk (*Cervus canadensis*) and free-ranging white-tailed deer in Minnesota and Wisconsin, respectively. Since then, CWD has been detected in 12 captive cervid facilities and 110 free-ranging deer in Minnesota (>90,000 tested), with the disease potentially established endemically in the southeast region, albeit at a low prevalence (La Sharr et al. 2019). Surveillance efforts primarily utilize samples from hunter-harvested animals along with those collected opportunistically from deer found dead in poor body condition, euthanized deer displaying clinical signs of CWD, deer killed by vehicle collisions, and targeted agency culling.

Diagnostic tests for CWD can be classified into two categories: first-generation antibody-based diagnostics such as immunohistochemistry (IHC) and enzyme-linked immunosorbent assay (ELISA) and second-generation prion protein amplification assays such as real-time quaking-induced conversion (RT-QuIC), and protein misfolding cyclic amplification. Management agencies have employed ELISA screening of medial retropharyngeal lymph nodes with confirmatory IHC on samples considered suspect by ELISA, because these have traditionally been considered the gold standard for CWD (Haley and Richt 2017). Both ELISA and IHC, the currently available validated assays for CWD, must be completed at National Animal Health Laboratory Network laboratories. This diagnostic bottleneck has resulted in laboratories being at or beyond testing capacity (Schuler et al. 2021). However, advances in CWD diagnostics allow the implementation of new diagnostic standards and sampling techniques, a critical development as testing pressures and expectations increase (Haley and Richt 2017; McNulty et al. 2019; Bloodgood et al. 2021; Henderson et al. 2020). Amplification assays have been refined over the past decade and show potential as the next gold standard choice of diagnostic tools with increased sensitivity in a high-throughput platform, as well as usefulness with nontraditional sample types such as blood, saliva, feces, and ear biopsies (Elder et al. 2015; Haley and Richt 2017; Tennant et al. 2020; Ferreira et al. 2021).

Few studies have compared the use of prion amplification assays with current immunodetection assays on free-ranging cervids (Haley et al. 2014). We aimed to compare the CWD detection capabilities of RT-QuIC test to ELISA using a population-level sample set of free-ranging deer from southeastern Minnesota.

MATERIALS AND METHODS

Study area and experimental design

The MNDNR contracted with US Department of Agriculture–Wildlife Services to conduct culling from 22 January to 29 March 2019 within areas of known CWD-positive deer detections near Preston and Winona, Minnesota; a karst topography region with mixed upland hardwoods, swamp, and agricultural lands (Fig. 1). Priority areas were designated spatially by Public Land Survey System sections (1 mi^2; 2.29 km^2) with a high number of total CWD-positive deer, positive female deer (considered to be disease anchors), or areas with high deer densities in close proximity to known positives. Intact carcasses were transported to the Preston Department of Natural Resources Forestry office where MNDNR staff collected tissue samples: medial retropharyngeal lymph nodes (RPLN), submandibular lymph nodes (SLN), parotid lymph nodes (PLN), palatine tonsils (PT), feces, whole blood, and neck muscles (not included in this study). Tissue collection tools (scalpel handles, forceps) were wiped of physical debris and cleaned with a bleach solution between animals. A new scalpel blade was used between animals. Collected tissues were handled with tools only and placed directly into 4-oz (118 mL) Whirl-Pak (Nasco, Fort Atkinson, Wisconsin, USA) bags. Blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube as a free flow at time of decapitation. Feces were collected from the anus by hand with new disposable gloves and placed directly into 4-oz Whirl-Pak bags. In some cases, significant coagulation of blood or lack of feces precluded collection. All samples were preserved at –20 C in the field.

In accordance with surveillance, RPLN samples from all deer were tested by CWD ELISA at Colorado State University Veterinary Diagnostic Laboratory (CSU VDL) using the Bio-Rad TeSeE Short Assay Protocol (SAP) Combo Kit (BioRad Laboratories Inc., Hercules, California USA). For each animal, a homogenate was produced using three subsamples from each RPLN and tested as a

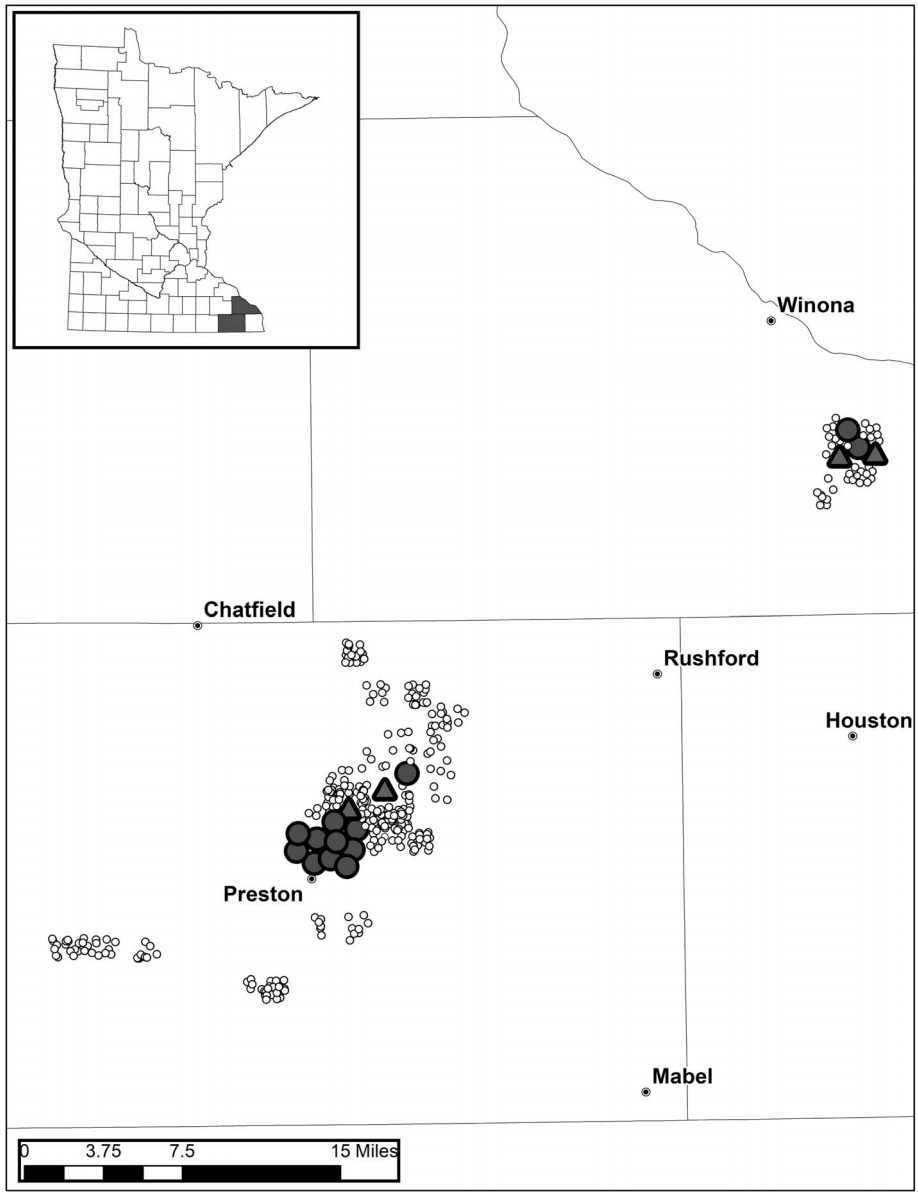

FIGURE 1. Locations of the 519 white-tailed deer (*Odocoileus virginianus*) culled from 22 January to 29 March 2019 by the Minnesota Department of Natural Resources near Preston and Winona, Minnesota, USA. Thirteen deer identified as chronic wasting disease (CWD) enzyme-linked immunosorbent assay, immunohistochemistry, and real-time quaking-induced conversion (RT-QuIC) positive by medial retropharyngeal lymph node samples are indicated by large circles. Four additional deer identified as CWD putative positives by RT-QuIC are indicated by large triangles.

pooled sample of both RPLN subsamples. Any suspect-positive RPLN from ELISA was confirmed through IHC of the prion protein as described (Hoover et al. 2016). The same procedures were used for all ELISA lymphoid tissue testing at CSU VDL.

RT-QuIC analysis

Following transport from the field, bilateral PLN, SLN, and PT, blood, and feces were preserved at –80 C until RT-QuIC analysis. All RT-QuIC research staff were blinded to the RPLN ELISA and IHC results.

Animal and sample identification: RT-QuIC screening began with sampling and testing of one PLN from each of the 519 deer. Samples that exhibited amyloid seeding activity (ASA) were tested a second time to confirm the laboratory procedures and both results were reported to the MNDNR. Due to limited resources, a 60-animal subset of the original 519 animals was created by MNDNR staff for further testing. This subset provided the following: 1) three times as many known negative animals as known positive animals (by ELISA testing of RPLN); 2) 300 individual samples across six sample types; and 3) nearly 1,800 individual RT-QuIC reactions. The subset included the following: 1) all deer that were CWD-positive by ELISA and IHC on RPLN; 2) all deer that exhibited ASA by RT-QuIC on unilateral PLN; and 3) a randomly chosen set of deer that were CWD not detected by ELISA on RPLN to reach 60 animals. In light of the study by Bloodgood et al. (2021) in which unilateral sampling of RPLN was less sensitive than bilateral sampling of RPLN, the 60-sample subset were subjected to RT-QuIC testing following bilateral sampling of PLN, SLN, and PT, as well as available whole blood and feces. Tissue samples were tested as a pool of tissue type per animal (e.g., bilateral PLN subsamples pooled). Within this sample subset, the 13 ELISA and IHC CWD-positive RPLN were provided by CSU VDL and tested by RT-QuIC. After samples were unblinded, bilateral PLN, SLN, and PT subsamples from all deer that exhibited ASA on at least one tissue type by RT-QuIC were provided to CSU VDL for ELISA-only testing. Note that the BioRad ELISA is only validated for retropharyngeal lymph nodes and obex. Staff at CSU VDL were blinded to the RT-QuIC results.

Substrate preparation: Recombinant hamster PrP (HaPrP90-231; provided by National Institutes of Health Rocky Mountain Laboratory) was cloned into the pET41a(+) expression vector and was expressed in Rosetta™ (DE3) *Escherichia coli* cells (Millipore Sigma, Darmstadt, Germany). Expression and purification of the recombinant substrate was performed following a modified version of the protocol from Orrù et al. (2017). Specifically, protein expression was induced using 0.75 mM isopropylthio-β-galactoside in place of Overnight Express™ autoinduction (Novagen, Darmstadt, Germany), and cells were cracked with two passes at 16,000 psi on a microfluidizer rather than using a homogenizer.

Lymph tissue preparation: Lymph tissue (RPLN, PLN, SLN, and PT) dissections were initiated by a cross-sectional cut with sample collection along the cut face to reduce potential cross-contamination that might have originated during field collection. We used disposable forceps and scalpels and surface decontamination between samples (1:1.5; 5.25% sodium hypochlorite). Samples were dissected on fresh disposable benchtop paper. A 10% (w:v) suspension was made by adding 100 mg of tissue to 900 µL of phosphate buffered saline (PBS). In the case of bilateral samples, 50 mg of each tissue was dissected and added to one tube. Tissue suspensions were homogenized using 1.5-mm-diameter zirconium oxide beads (Millipore Sigma, Burlington, Massachusetts, USA) and a BeadBug homogenizer (Benchmark Scientific, Sayreville, New Jersey, USA), maximum speed for 90 s. Homogenized samples were stored at –80 C. Samples were diluted further to 10^{-3} in dilution buffer (0.1% sodium dodecyl sulfate, 1× PBS, N-2 Supplement [Life Technologies Corporation, Carlsbad, California, USA]), and 2 µL were added to 98 µL of RT-QuIC master mix. The final tissue dilution factor in the reaction was 1:50,000.

Blood preparation: We modified a protocol for blood preparation from Elder et al. (2015) and the phosphotungstic acid precipitation was first described by Safar et al. (1998). One mL of EDTA whole blood was placed in a tube with 1.5-mm-diameter zirconium oxide beads and underwent four cycles of flash freeze-thaw consisting of 3 min in dry ice and 3 min at 37 C. It was then homogenized using a BeadBug homogenizer at top speed for two cycles (a total of 180 s). The homogenate was centrifuged at 500 × G for 2 min. We incubated 100 µL of supernatant with 7 µL of 4% (w/v) phosphotungstic acid (Sigma-Aldrich, St. Louis, Missouri, USA) in 0.2 M magnesium chloride. The product was then incubated in a ThermoMixer (Eppendorf, Enfield, Connecticut, USA) at 37 C for 1 h (1,500 rpm) and subsequently centrifuged for 30 min at 21,100 × G. The pellet was resuspended in 20 µL of dilution buffer. We added 2 µl of the 10^{-2}–diluted suspension to the RT-QuIC reaction described next.

Feces preparation: We modified a fecal preparation protocol developed by Tennant et al. (2020). The fecal pellet was manually homogenized into 10% homogenates using 1× PBS. The solution was centrifuged at 1,000 × G for 15 min at 4 C. We centrifuged 500 µL of supernatant at 21,100 × G for 30 min. The pellet was resuspended in 100 µL of 1× PBS and incubated with 7 µL of phosphotungstic acid solution as described earlier, then centrifuged for 30 min at 21,100 × G. The pellet was resuspended in 10 µL 0.1% sodium dodecyl sulfate in PBS. We added 2 µL of this suspension to the RT-QuIC reaction described herein.

Assay parameters: Recombinant hamster PrP substrate was filtered at 3,000 × G through a 100 kDa molecular weight cutoff spin column. A master mix was made to the following concentrations: 1× PBS, 500 µM EDTA, 50 µM Thioflavin

T, 300 mM NaCl, and 0.1 mg/mL rPrP. All reagents were filter-sterilized through 0.22-μm polyvinyl difluoride membrane filters. We pipetted 98 μL of the master mix into each well on a black 96-well plate with clear bottoms. Twelve to 16 wells were used for controls: four to six with CWD-negative WTD lymph nodes, four to six with CWD-positive WTD lymph nodes, and four with no sample. Plates were sealed with clear tape then shaken on a BMG FLUOstar® Omega microplate reader (BMG LABTECH Inc., Cary, North Carolina, USA) at 700 rpm, double orbital for 57 s, then rested for 83 s, repeated 21 times, then the fluorescence was recorded. For all tissue types, the temperature was set to 42 C. The whole shake-and-rest then read cycle was repeated 58 times for a total of about 46 h. Readings were recorded with an excitation filter of 450 nm and an emission filter of 480 nm. The gain was set to 1,600. The machine performed 21 flashes per well.

RT-QuIC data analysis: Rate of amyloid formation was determined as the inverse of hours (1/h) for ASA to surpass a threshold of 10 standard deviations above the average baseline readings after 4.5 h for each plate (Hoover et al. 2016). A minimum of four replicates were performed for each sample. Samples were considered an indeterminate (putative positive) result when at least 50% of the replicates gave a fluorescence signal higher than the threshold cut-off value. *P* values were calculated based on rate of amyloid formation versus negative controls using a Mann-Whitney *U*-test as described by Tennant et al. (2020) to determine the statistically significant difference in rate of amyloid formation between samples tested and negative controls on the respective plates. Statistical significance was established at 0.05 (α=0.05) and *P* values below 0.05 were considered statistically different. We additionally examined statistical significance for all RT-QuIC data using a maxpoint ratio analysis (Vendramelli et al. 2018; see Supplementary Methods). All statistical analyses were performed using GraphPad Prism software (version 9, San Diego, California, USA). We report 95% Wilson score confidence limits for proportions, which is appropriate for small sample size and sample proportions close to 0 or 1 (Brown et al. 2001). Confidence limits were estimated using Epitools Epidemiological Calculators (Ausvet, Bruce, Australian Capital Territory, Australia).

RESULTS

First generation surveillance: ELISA

Of the 519 culled deer, RPLN from 13 deer (0.025; 95% Wilson confidence limits [CL], 0.015–0.042) were reported as CWD positive by CSU VDL through ELISA screening and IHC confirmation (Fig. 1 and Table 1).

Second generation surveillance: RT-QuIC

The first objective for RT-QuIC analysis was a blinded screening of the 519 deer using unilateral PLN samples. The first analysis identified 11 (0.021; 95% CL, 0.012–0.038) samples exhibiting significant ($P<0.05$) ASA with repeated results upon retesting (Table 1). Of the approximately 4,150 individual RT-QuIC reactions underlying these 519 PLN samples, we observed significant ASA within the PLN samples of the 11 deer noted earlier and in a single well (nonsignificant) in only 11 other PLN samples. We next examined the diagnostic agreement between these 11 animals with significant ASA, animals previously classified as CWD-positive by ELISA and IHC (n=13), and a series of ELISA-negative controls (total n=60). One animal that was positive by ELISA and IHC (MN100528) was mistakenly not included in the 60-deer sample set, but had the same tissue set screened subsequently (following unblinding) bringing the sample set to 61 deer.

The RT-QuIC results indicated 16 (26%) of 61 deer exhibited significant ($P<0.05$) ASA in at least one sample type. We observed ASA in the following: 12/61 (0.20; 95% CL, 0.12–0.31) bilateral PLN, 12/61 (0.20; 95% CL, 0.12–0.31) bilateral SLN, 13/61 (0.21; 95% CL, 0.13–0.33) bilateral PT, 13/13 (1.0; 95% CL, 0.77–1.0) bilateral RPLN, 7/51 (0.14; 95% CL, 0.07–0.26) whole blood, and 5/47 (0.11; 95% CL, 0.05–0.23) feces (Table 1 and Fig. 2). Sample prevalences estimated from this subset and the original 519 are biased toward CWD-positive detections (originating from deer social groups in areas of documented CWD cases) and do not reflect the true population prevalence.

We observed intraindividual variability in the types of tissues that exhibited ASA for each of the 16 animals (Figs. 2, 3). We detected ASA beneath levels of significance ($P<0.05$) in lymphoid and blood samples (n=6) from several of these 16 animals and considered them as indeterminate results

TABLE 1. Samples from a 61 white-tailed deer (*Odocoileus virginianus*) subset of the 519 deer culled from 22 January to 29 March 2019 by the Minnesota Department of Natural Resources (DNR) near Preston and Winona, Minnesota, USA. Samples listed are from deer that indicated chronic wasting disease enzyme-linked immunosorbent assay (ELISA) positivity and/or exhibited amyloid seeding activity (ASA) by real-time quaking-induced conversion (RT-QuIC) on at least one sample type. LN = lymph node; X = statistically significant ASA determined by Mann-Whitney *U*-test and Dunnet's test ($P<0.05$; see Supplementary Methods); ND = not detected; O = none or not statistically significant ASA determined by Mann-Whitney *U*-test and Dunnet's test ($P<0.05$; see Supplementary Methods); — = data not available because samples were not available.

		Unilateral parotid LN		Bilateral medial retropharyngeal LN		Bilateral parotid LN		Bilateral submandibular LN		Bilateral palatine tonsil			
DNR ID	Sex, age	RT-QuIC 1st run	RT-QuIC 2nd run	RT-QuIC	ELISA	RT-QuIC	ELISA	RT-QuIC	ELISA	RT-QuIC	ELISA	Blood	Feces[a]
MN137315	Female, adult	X	X	X	3.999	X	3.263	X	3.999	X	3.999	O[b]	O
MN137450	Male, yearling	X	X	X	3.999	X	3.999	X	3.999	X	3.999	X	O
MN106838	Male, adult	X	X	X	3.999	X	0.431	X	2.006	X	3.999	—	—
MN145282	Female, adult	X	X	X	3.999	X	3.999	X	3.999	X	3.999	X	X
MN137289	Female, adult	X	X	X	3.999	X	3.390	X	3.999	X	3.999	X	X
MN137471	Female, adult	X	X	X	3.999	X	0.167	X	3.999	X	3.999	X	X
MN137479	Female, adult	X	X	X	3.999	X	0.310	X	3.999	X	3.999	X	O
MN137193	Male, adult	X	X	X	3.999	X	3.999	X	3.035	X	3.999	O[c]	O
MN101141	Female, adult	X	X	X	3.999	X	0.320	X	3.999	X	3.999	O[c]	—
MN145279	Female, adult	X	X	X	3.999	X	3.999	X	3.999	X	3.999	X	O
MN137293	Female, adult	X	X	X	3.287	X	3.189	X	3.999	X	3.999	X	X
MN100528	Female, adult	O	O	X	3.188	O	ND	O[b]	ND	X	0.468	O	—
MN137219	Male, adult	O	O	X	1.315	O	ND	O	ND	O[b]	ND	O	O
MN137346	Male, fawn	O	O	—	ND	O[b]	ND	O	ND	O	ND	—	X
MN137203	Male, yearling	O	O	—	ND	X	0.311	O	ND	O	ND	O[b]	O
MN145273	Male, adult	O	O	—	ND	O	ND	O	ND	X	ND	O	O
MN145287	Female, adult	O	O	—	ND	O	ND	O	ND	O[b]	ND	O	O

[a] Best practices for RT-QuIC on fecal samples indicate fresh collection (at time of defecation or death) and flash freezing for optimal prion amplification detection. These conditions were not reproduced in this study (Tennant et al. 2020).

[b] Results demonstrate at least 50% of wells exhibited ASA by RT-QuIC but were not statistically significant by Mann-Whitney *U*-test and Dunnet's test (indeterminate result; $P<0.05$; see Supplementary Methods).

[c] Blood sample coagulated. Noncoagulated blood should be used for RT-QuIC prion detection (Elder et al. 2015).

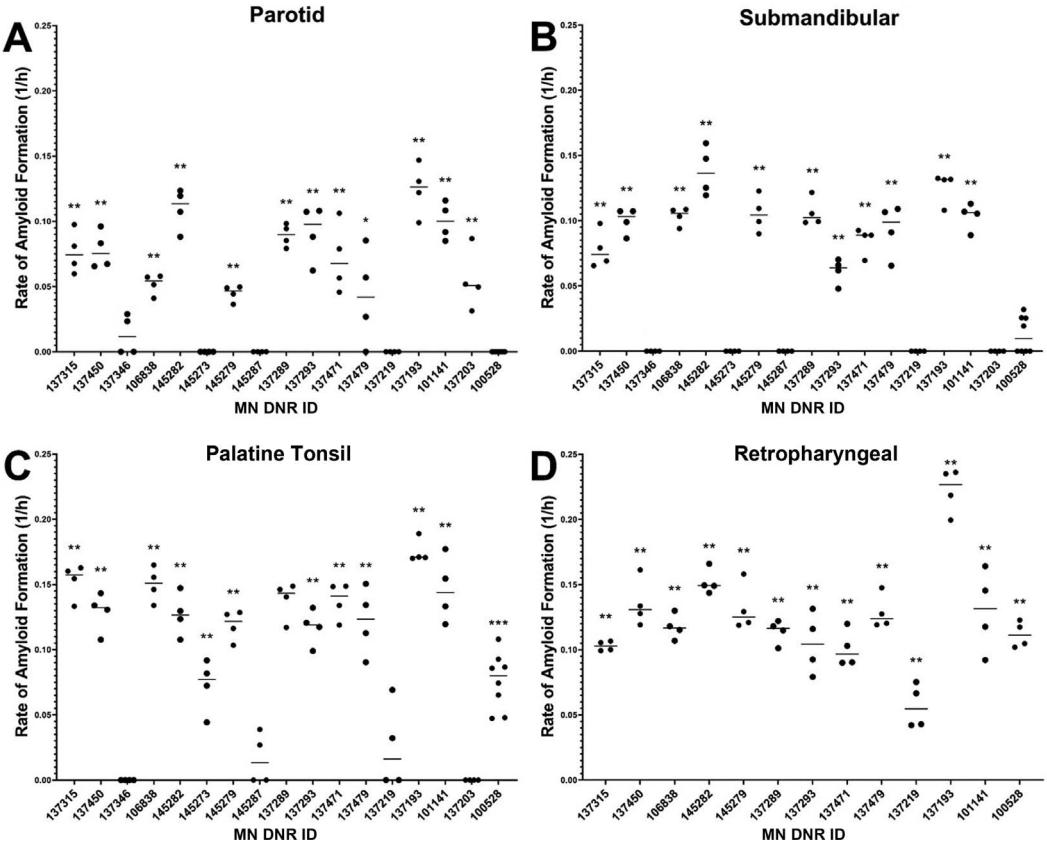

FIGURE 2. Relative rates of amyloid formation (1/h) demonstrated through real-time quaking-induced conversion of lymphoid tissue samples collected from a 61 white-tailed deer (*Odocoileus virginianus*) subset of the 519 deer culled from 22 January to 29 March 2019 by the Minnesota Department of Natural Resources (MN DNR) near Preston and Winona, Minnesota, USA. Samples exhibiting amyloid seeding activity were deemed positive by Mann-Whitney *U*-test and Dunnet's test (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; see Supplementary Methods). Parotid=parotid lymph node; submandibular=submandibular lymph node; retropharyngeal=medial retropharyngeal lymph node.

(Table 1 and Fig. 2). Additionally, amyloid seeding was detected in 2/4 replicates (indeterminate result; not significant) within a palatine tonsil sample from a single animal (MN145287; adult, female) that had no indication of ASA in any other sample type and was ELISA negative across all sample types (Table 1 and Fig. 2). No other samples from the 61 deer demonstrated ASA.

ELISA analysis of animals exhibiting ASA by RT-QuIC

To provide tissue-specific comparison between ELISA and RT-QuIC, PLN, SLN, and PT samples (n=51) from the 17 RT-QuIC positive or indeterminate animals were blind-tested by ELISA at CSU VDL. Fifty of the 51 samples demonstrated RT-QuIC and ELISA results agreement (Table 1). Deer MN145273 PT exhibited significant ($P<0.05$) ASA by RT-QuIC and was not detected by ELISA. Four samples (MN100528 SLN, MN137219 PT, MN137346 PLN, MN145287 PT) demonstrated indeterminate ASA (beneath levels of significance) and were not detected by ELISA. Samples positive on both testing platforms showed a moderate positive correlation of the semiquantitative measures of prion content (R^2=0.581; Fig. 4). Samples

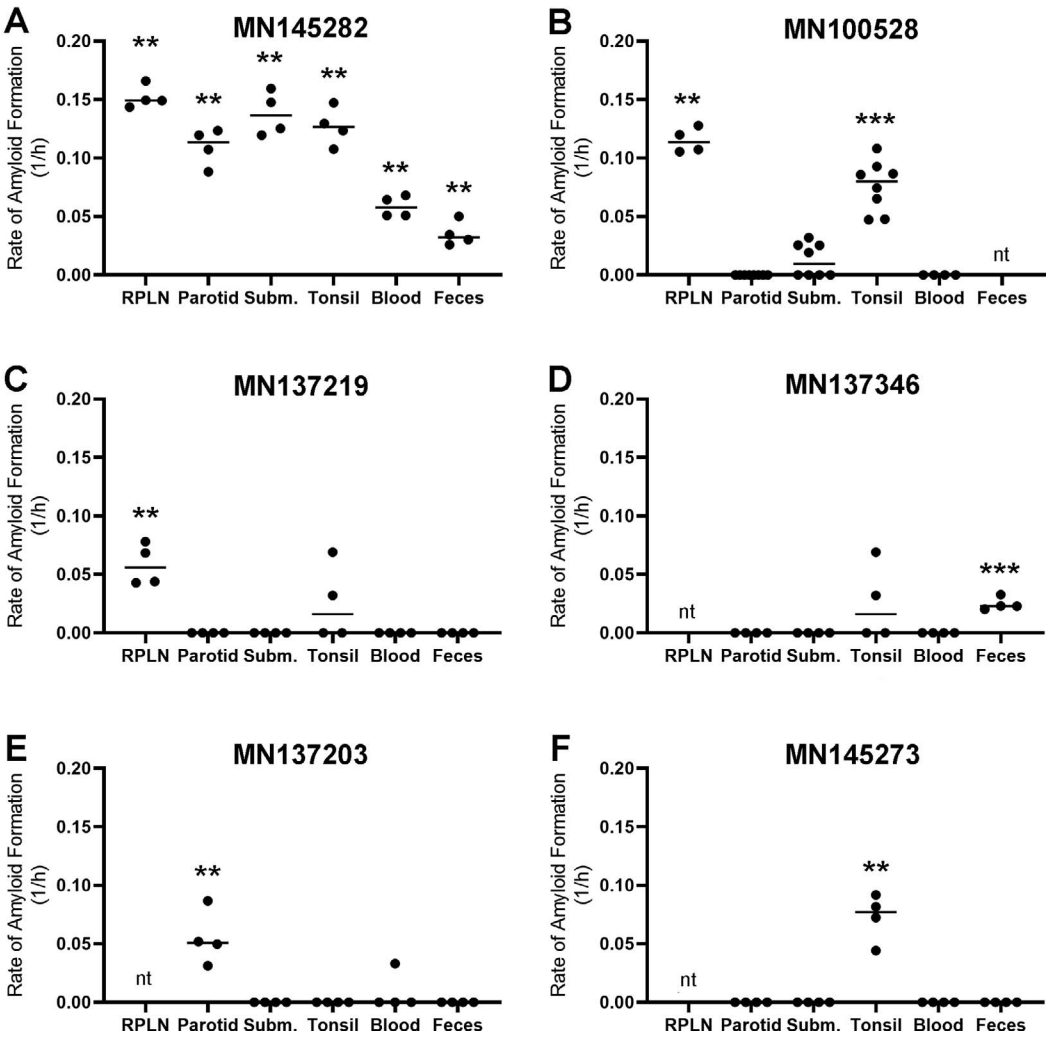

FIGURE 3. Relative rates of amyloid formation (1/h) demonstrated through real-time quaking-induced conversion (RT-QuIC) of all sample types from six of the 519 white-tailed deer (*Odocoileus virginianus*) culled from 22 January to 29 March 2019 by the Minnesota Department of Natural Resources near Preston and Winona, Minnesota, USA. Note the variability in amyloid seeding activity (ASA) across animals. (A) demonstrates a highly CWD-positive animal across all sample types. (B) is probably an animal in earlier disease progression when compared to (A). (C–F) are probably animals with very early infection in that only one sample type is statistically significant. Note that the deer shown in (D–F) did not have medial retropharyngeal lymph nodes (RPLN) available to test because they were not detected as CWD-positive by enzyme-linked immunosorbent assay and thus had been discarded at Colorado State University Veterinary Diagnostic Laboratory. Samples exhibiting ASA were deemed positive by Mann-Whitney *U*-test and Dunnet's test (***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; see Supplementary Methods). nt=not tested because those samples were not available for RT-QuIC testing; parotid=parotid lymph node; RPLN=medial retropharyngeal lymph node; subm.=submandibular lymph node; tonsil=palatine tonsil.

with an ELISA optical density (OD) value of 3.999 did not bias the correlation, because a similar R^2 was derived from the sample set with those samples removed (data not shown).

DISCUSSION

The goal for diagnostic assays is to optimize target detection so that animals that are diseased or infected are identified as true

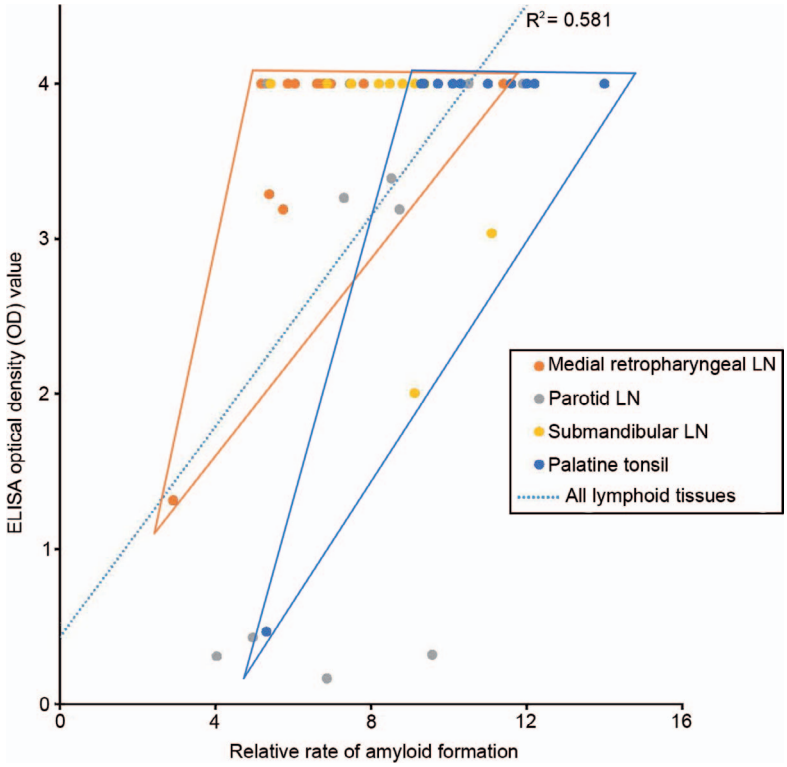

FIGURE 4. Relationship between the relative rate of amyloid formation (1/h) demonstrated through real-time quaking-induced conversion (RT-QuIC) and enzyme-linked immunosorbent assay (ELISA) optical density (OD) for lymphoid tissues examined from a 61 white-tailed deer (*Odocoileus virginianus*) subset of the 519 deer culled from 22 January to 29 March 2019 by the Minnesota Department of Natural Resources near Preston and Winona, Minnesota, USA. Depicted samples revealed both amyloid seeding activity by RT-QuIC and had OD values between 0.100 and 3.999. A moderate positive correlation is appreciated across all lymphoid tissue samples (dotted line; Pearson's correlation coefficient, R^2=0.581). The relationship between medial retropharyngeal lymph node (RPLN) and palatine tonsil (PT) samples is appreciated in that RPLN samples had a generally lower rate of amyloid formation than PT samples. LN=lymph node.

positives (sensitivity) and animals that are not diseased are identified as true negatives (specificity). An array of factors affects this optimization including, but not limited to, sample type and integrity, sampling method, assay elements, and individual animal (e.g., physiologic or immune state, genetics) and population characteristics (e.g., pathogen prevalence). For CWD, disease detection in wild populations has primarily been based on ELISA and IHC and the use of postmortem RPLN and/or obex samples. This approach might be limited with respect to the detection of low levels of prion burden as seen in early stages of disease (Hoover et al. 2016; Haley and Richt 2017; Hoover et al. 2017; McNulty et al. 2019). Our results support RT-QuIC's potential to detect low levels of CWD prion by using a diverse sample set (multiple lymphoid tissues, blood, and feces) that might have value for earlier detection of the disease.

A growing body of research collectively provides evidence that second-generation amplification-based assays have greater sensitivity in the detection of misfolded PrP than first-generation tests (Hoover et al. 2016; Haley and Richt 2017; Soto and Pritzkow 2018; McNulty et al. 2019). Our results support this conclusion as varied tissue types exhibited ASA with RT-QuIC, where, in some cases, ELISA testing resulted in no detection of PrP^{CWD} in the specific tissue type or within

the animal. This led to the discovery of four putative CWD-positive animals by RT-QuIC out of a pool of 48 deer with CWD not detected by ELISA on RPLN, as well as additional putative positive sample types from two deer that were CWD positive by ELISA on RPLN. Collectively, these six animals demonstrated intra-animal variability in CWD-positive tissues (ELISA and/or RT-QuIC). Based on relatively low ELISA OD values of the positive tissues in these six animals, and given that RT-QuIC identified tissues that exhibited ASA that were not detected by ELISA, we posit that these six animals were either in the early stages of CWD infection or represent RT-QuIC false-positives. We believe that the former is the case because zero ASA was observed in the 44 "negative" animals (i.e., zero of four replicates) and there was no indication of inter-animal or intra-animal cross-contamination from the sample collection, preparation, or RT-QuIC processes based on the spatial and chronological distribution of CWD detections and the reliability of plate-level controls. It is also possible that these six animals exhibited natural CWD prion strain variability to the degree that effective antibody-based detection via ELISA was hampered (discussed soon).

It is problematic to confirm the status of RT-QuIC-positive samples that are negative by first-generation methods because amplification assays approach attogram levels of misfolded prion protein detection, multifold lower than the limits of immunodetection methods (Haley et al. 2018b). However, similar studies focused on longitudinal analyses of CWD infection support claims that amplification-based assays can effectively detect CWD infections prior to both ELISA and IHC (Haley et al. 2018a, 2020; Denkers et al. 2020; Henderson et al. 2020). Previous studies also indicate that the unequal tissue distribution of CWD prion protein in early stages of the disease, coupled with sectioning or sampling technique variability (concerns with detection assays in general), contributes to the apparent reduced sensitivity of ELISA and IHC (Hoover et al. 2017; Bloodgood et al. 2021). Another complicating factor when comparing first- and second-generation CWD assays is the potential for variable strains of PrP^{CWD} to be detected by RT-QuIC or protein misfolding cyclic amplification but missed by immunodetection methods due to strain sensitivity to enzymatic digestion. The ELISA (BioRad TeSeE specifically), IHC, and western-blotting methods use antibodies that do not distinguish between PrP and PrP^{CWD}, necessitating Proteinase-K digestion to identify PrP strains resistant to degradation. Recent data indicate that a variety of PrP^{CWD} strains are circulating in cervids, raising the possibility that diagnostic methods not using enzymatic digestion have greater potential for identifying a broader family of PrP^{CWD} (Duque Velásquez et al. 2015; Osterholm et al. 2019). Simultaneously, the potential for naturally occurring, noninfectious conformations of healthy PrP to cause false-positives in amplification-based analyses must be considered.

Sample type also influences CWD test sensitivity, particularly early in infection. Amyloid seeding activity has been documented by RT-QuIC in feces and blood of experimentally infected deer in the preclinical stages and shortly after inoculation, respectively (Elder et al. 2015; Tennant et al. 2020). In both studies, optimal sample conditions—limiting freeze-thaw of feces and heparin preservation of blood—presented the most consistent results. We did not have these ideal conditions, which might have contributed to the variable RT-QuIC results from feces and blood. The pathogenesis of CWD provides valuable insight into the prion seeding activity we observed in PT. Tonsillar tissue is one of the first tissues to demonstrate prion immunodeposition, which fits with observed prion traffic through the lymphatic system and is supported in this study (Haley and Richt 2017; Hoover et al. 2017; Henderson et al. 2020). Palatine tonsil tissue from multiple animals, possibly in early stages of the disease, exhibited ASA by RT-QuIC and had either low OD values or were not detected by ELISA. This might be a limitation of the application of ELISA to this particular tissue type; the BioRad ELISA is only validated for

retropharyngeal lymph nodes and obex. Either way, our results support the value of PT as a tissue of choice for CWD surveillance, particularly when using RT-QuIC. Based on our results, PT demonstrated higher levels of ASA (i.e., "hotter" samples) than did RPLN, yet a majority of both sample types were at the upper limits of detection by ELISA. This leads to the possibility that although RPLN can be good for ELISA-based surveillance, PT might be the ideal tissue type for RT-QuIC-based surveillance. Although the feasibility of identifying PT versus RPLN in field extraction should be considered, further investigation is warranted. We also document the potential for RT-QuIC screening of PLN to identify early stages of CWD infection, and our results support the continued importance of RPLN for CWD surveillance. Potential limitations to these results lie in the sampling scheme of this study. Although RT-QuIC and ELISA were performed on the same tissue types across and within animals, different subsamples of each tissue were used for each assay. Regardless, there was a high-level of correspondence between the testing methods. Collectively, our RT-QuIC data provide evidence that multitissue sampling would enhance detection and removal of CWD-positive animals through postmortem CWD surveillance, although fiscal limitations must be considered when considering sample type(s) and testing protocols beyond the current standards.

Early outbreak detection through CWD surveillance is imperative. This is particularly true for geographic locations where CWD has not been detected previously, because earlier disease discovery on the landscape leads to earlier implementation of management and more effective control (Miller and Fisher 2016). The benefit of enhanced test sensitivity is a reduction in the sample number needed for disease detection and monitoring, particularly when population prevalence is low. We posit that the growing number of studies documenting the utility of RT-QuIC for the surveillance of a variety of protein-misfolding and prion diseases, including CWD, collectively demonstrate that the method is robust and will greatly aid our understanding and control of CWD (Wilham et al. 2010; Orrú et al. 2015; Caughey et al. 2017; Franceschini et al. 2017; Cooper et al. 2019; Saijo et al. 2019; Henderson et al. 2020; Rossi et al. 2020). Our study provides additional data on RT-QuIC as the assay is further developed and validated for future CWD surveillance, management, and regulatory initiatives.

Future investigation, building on these discoveries, will include optimization of a sampling protocol for CWD amplification assays to increase detection sensitivity, elucidating potential impacts of prion protein allele variation on testing for particular CWD strains, and continued analyses of multitissue samples secured from CWD-positive regions. These efforts, combined with robust epidemiological validation to characterize diagnostic test performance (sensitivity and specificity) of new CWD diagnostic tests will help to establish a more complete depiction of the CWD landscape. Our study, using a blinded sample set of free-ranging WTD from documented CWD hotspots, and multiple sample types, indicate that RT-QuIC might be a more powerful tool than current methods in identifying CWD-positive animals in coordination with a multitissue sample collection protocol, particularly for early infections and in previously CWD-free locations, whether free-ranging or farmed. These findings have direct implications for the effective surveillance and management of CWD across all cervids.

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SUPPLEMENTARY MATERIAL

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Evaluation of Real-Time Quaking-Induced Conversion, ELISA, and Immunohistochemistry for Chronic Wasting Disease Diagnosis

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Chronic wasting disease (CWD) is a transmissible prion disorder, primarily affecting free-ranging and captive cervids in North America (United States and Canada), South Korea, and Europe (Finland, Norway, and Sweden). Current diagnostic methods used in the United States for detection of CWD in hunter harvested deer involve demonstration of the causal misfolded prion protein (PrP^{CWD}) in the obex or retropharyngeal lymph nodes (RLNs) using an antigen detection ELISA as a screening tool, followed by a confirmation by the gold standard method, immunohistochemistry (IHC). Real-time quaking-induced conversion (RT-QuIC) assay is a newer approach that amplifies misfolded CWD prions *in vitro* and has facilitated CWD prion detection in a variety of tissues, body fluids, and excreta. The current study was undertaken to compare ELISA, IHC, and RT-QuIC on RLNs ($n = 1{,}300$ animals) from white-tailed deer (WTD) in Michigan. In addition, prescapular, prefemoral and popliteal lymph nodes collected from a small subset ($n = 7$) of animals were tested. Lastly, the location of the positive samples within Michigan was documented and the percentage of CWD positive RLNs was calculated by sex and age. ELISA and RT-QuIC detected PrP^{CWD} in 184 and 178 out of 1,300 RLNs, respectively. Of the 184 ELISA positive samples, 176 were also IHC positive for CWD. There were seven discordant results when comparing IHC and ELISA. RT-QuIC revealed that six of the seven samples matched the IHC outcomes. One RLN was negative by IHC, but positive by ELISA and RT-QuIC. RT-QuIC, IHC, and ELISA also detected PrP^{CWD} in prescapular, prefemoral and popliteal lymph nodes. CWD infection heterogeneities were observed in different age and sex groups, with young males having higher CWD prevalence. All, except one, CWD positive RLNs analyzed were from ten Counties geographically located in the West Michigan region of the Lower Peninsula. Taken together, we show evidence that the RT-QuIC assay is comparable to ELISA and IHC and could be helpful for routine CWD detection in surveillance programs. RT-QuIC also demonstrated that CWD prions are distributed across lymph nodes in a variety of anatomic locations. A multi-laboratory

validation on blinded sample panels is underway and is likely to help to provide insight into the variability (lab-to-lab), analytical sensitivity, and specificity of gold standard diagnostics vs. RT-QuIC assay.

Keywords: deer, prions, RT-QuIC, immunohistochemistry, diagnostics, chronic wasting disease (CWD)

INTRODUCTION

Chronic wasting disease (CWD) is the only known transmissible spongiform encephalopathy to occur in free-ranging wildlife populations, naturally infecting elk, moose, and various deer species. To date, the disease has been reported in 26 American states, four Canadian provinces, South Korea, Norway, Finland, and Sweden (1–5). It continues to spread across North America through new and ongoing outbreaks. The exact mechanisms of CWD spread remains unclear. Direct contact with saliva, urine, feces, or aerosols from infected animals, as well as indirect (environmental) contact through the ingestion of infectious prions bound to soil or plants, and vertical transmission, likely contribute to disease spread (6–8). Mathematical models of CWD prevalence and dynamics support the hypothesis that direct transmission accounts for most transmission events, with both population density and contact frequency contributing to CWD spread (9, 10). However, the extraordinary stability and resistance to degradation of the CWD prion allows it to remain infectious in the environment for several years (6, 11). Furthermore, 18 to 20 months may pass between initial infection and the onset of clinical signs. The long incubation period contributes to CWD transmission as asymptomatic infected animals can substantially contaminate the environment through body secretions such as urine, saliva, and feces (12–14). However, animals in the first stages of the disease contain concentration of prions in their tissues that cannot be detected by standard methods such as ELISA or immunohistochemistry (IHC).

As a prion disease, CWD is caused by a pathogenic, misfolded conformation of the normal, natively folded, cellular prion protein to a pathogenic prion conformer PrP^{CWD}, which accumulates mostly in the central nervous system (15–18). During the infection, PrP^{CWD} propagates via a process of seeded polymerization, characterized by increased formation of β-sheets, propensity to aggregate into amyloid fibrils, and resistance to protease and acid digestion (16, 19–21). Definitive diagnosis of CWD in the United States relies on the detection of PrP^{CWD} in the brain or retropharyngeal lymph nodes (RLNs) by federally recognized diagnostic methods performed at approved laboratories: ELISA and IHC. Despite their reliability, such methods have limited sensitivity and application across various tissues and body excreta in comparison to *in vitro* amplification methods for prion detection, such as protein misfolding cyclic amplification and real-time quaking-induced conversion (RT-QuIC) assays. RT-QuIC assay offers a newer, high-throughput approach that can detect minute amounts of prions in tissues and secretions of CWD-infected animals with the additional advantage of avoiding the proteolytic or acidic pretreatments commonly required for the conventional CWD detection assays (ELISA and IHC). Even though RT-QuIC has been reported to efficiently amplify PrP^{CWD} in a variety of tissues (e.g., RLNs, brain, ear pinna, third eyelid, muscle), body fluids (e.g., nasal swabs, CSF, blood), and excreta (e.g., saliva, urine, feces) of animals at clinical and, sometimes, at preclinical stages of disease, the method is not yet approved for official CWD surveillance programs (13, 22–30). Nevertheless, sensitive and rapid detection of low levels of prions in early stages of infection would aid substantially in understanding the epidemiology of the disease and enable assessment of mitigation based on management strategies to minimize disease transmission.

A few studies have investigated the utility of RT-QuIC in comparison to current immunodetection assays on free-ranging cervids (23, 28, 30). In the present study, the consistency of results between RT-QuIC, ELISA and IHC for the detection of CWD was analyzed in 1,300 RLNs of white-tailed deer (WTD, *Odocoileus virginianus*) collected between 2015 and 2021 in Michigan. Furthermore, the utility of RT-QuIC assay for the detection of CWD prions in a broader set of lymphatic tissues was examined. Lastly, the prevalence of CWD by age and sex was calculated and the location of the positive samples within Michigan documented. Our results demonstrate that males present two times higher prevalence than female WTD in Michigan. CWD infection seems to increase with age, but steadily declines after the fourth year of life. Our data also suggests that CWD is broadly present in lymph nodes of infected deer. Moreover, the RT-QuIC assay performance is comparable to conventional assays for CWD detection in lymph nodes of WTD in terms of sensitivity and it could be helpful as a new CWD diagnostic tool.

MATERIALS AND METHODS

Sample Collection

No animal-use-protocol approval was required for this study since all samples were procured from deer submitted for testing at the Michigan State University, Veterinary Diagnostic Laboratory (MSU-VDL). Anatomic location and number of lymph nodes tested on this work are shown in **Figure 1**. One thousand three hundred WTD RLNs, eight prescapular lymph nodes, eight popliteal lymph nodes, and six prefemoral lymph nodes were collected post-mortem by trained laboratory personnel from 2015 to 2021 as part of the CWD surveillance in Michigan. Portions of lymph node were stored at−20°C (for ELISA and

Abbreviations: CWD, Chronic wasting disease; IHC, Immunohistochemistry; MSU-VDL, Michigan State University, Veterinary Diagnostic Laboratory; NVSL, National Veterinary Services Laboratories; OD, optical density; PrP^{CWD}, misfolded prion protein; RLN, retropharyngeal lymph node; RT-QuIC, Real-time Quaking-Induced Conversion; WTD, White-tailed deer; yo, years old.

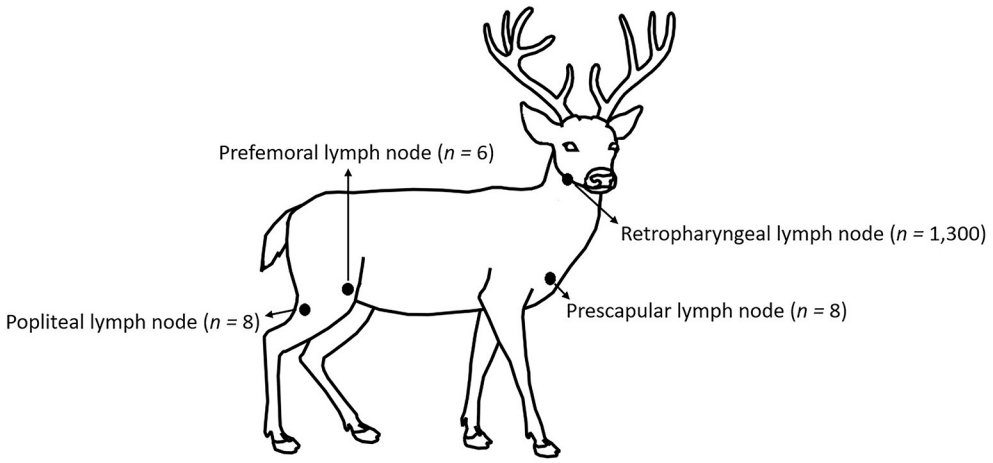


FIGURE 1 | Location and number of lymph nodes tested in this study.

RT-QuIC), while the remaining node was fixed in 10% neutral-buffered formalin (for IHC). Information about the location, sex, and age (estimation based on tooth replacement and wear) of the animals was also collected.

ELISA

The TeSeE™ Short Assay Protocol (SAP) Combi Kit (Bio-Rad) ELISA was performed following the manufacturer's instructions. Briefly, 2 or 3 wedge shaped sections of lymph node cortex with a total weight of 180 to 220 mg were placed into a grinding tube with lysis buffer and a large ceramic bead, all supplied in the SAP Combi Kit. The pieces of lymph node were homogenized using a FastPrep® (MP Biomedicals, Irvine, CA) bead-beater homogenizer for 2 cycles of 45 s at 6.5 m/sec. Next, 250 μl of homogenate from the grinding tube was placed into a 2 ml microcentrifuge tube, mixed with 250 μl of a proteinase K solution supplied in the Kit, and incubated at 35 to 39○C for 10 min. Then 250 μl of reagent B from the SAP Combi Kit was added to the microcentrifuge tube and mixed by inversion. Finally, the mixture was clarified by centrifugation for 7 min at 15,000 rcf at 20○C. The supernatant was removed, the pelleted material was air dried, and 25 μl of reagent C from the kit was added to the microcentrifuge tube. After a 5-min incubation at 95–105○C, the contents of the tube were cooled and then briefly vortexed. Except for the homogenization step, this process was repeated in duplicate for each sample that was non-negative in the ensuing ELISA. For the ELISA, 125 μl of sample diluent from the kit was added to the tube, which was then briefly vortexed and 100 μl of the resulting mixture was added to an ELISA plate well. When all samples had been added to the ELISA plate, the plate was covered with an adhesive shield and incubated at 37○C for 30 min. After incubation, the plate was washed following the manufacturer's recommendations and blotted dry before 100 μl of conjugate solution from the kit was added to each well of the plate. The plate was then incubated for 30 min at 2–7○C, washed, and blotted dry. Then 100 μl of chromogen substrate solution from the kit was added to each well and the plate was incubated for 30 min at 18–30○C. Finally, 100 μl of a stop solution from the kit was added to each well and a microplate reader with a 450 nm filter was used to measure the optical density (OD) of each well. The interpretation of the results followed the National Veterinary Services Laboratories (NVSL)-SOP-0883 which states that a sample is non-negative if the OD is greater than the mean of 4 negative control wells plus 0.035. Samples with results situated just below the cut-off value (cut-off value-10%), as well as samples with an OD greater than or equal to the cut-off value were retested in duplicate, starting from the original homogenate. After repeating the ELISA, a sample was considered positive if at least 2 measurements were non-negative, and it was then submitted for further testing using IHC.

Immunohistochemistry

All ELISA-positive and RT-QuIC-positive RLNs ($n = 184$) were tested by IHC at the MSU-VDL and by the NVSL. A sample was considered positive if IHC positive result was obtained from at least one of those laboratories. Another 188 randomly selected CWD negative RLN samples and prescapular lymph nodes, prefemoral lymph nodes, and popliteal lymph nodes ($n = 22$) were tested by IHC at the MSU-VDL. Formalin fixed lymph nodes were bisected, and tissue sections were incubated for 60 min with 95% formic acid followed by a 24-h rinse in tap water. Tissues were transferred into 10% neutral buffered formalin and after routine overnight deparaffinization, lymph node sections were embedded into paraffin blocks. Sections were cut at 5 to 7 μm thickness and slides were dewaxed, rehydrated, and treated in 95% formic acid for 5 min followed by multiple washes in Tris buffer at pH 7.5. Following antigen retrieval using the DAKO target retrieval solution (10X concentrate, S2369, Agilent, Santa Clara, CA) in a decloaker (BioCare Medical, Pacheco, CA), slides were immunohistochemically labeled for PrP^{CWD} following the NVSL protocol (SOP-PS-0002) (31, 32). Briefly, IHC was performed with the Ventana Discovery Ultra autostainer (Roche Diagnostics, Indianapolis, IN) that utilizes the kit-supplied F99 antibody (Anti-Prion Research

Kit, RTU, Cat# 760-231; Roche Diagnostics, Indianapolis, IN), a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromogen (fast red A, naphthol, fast red B), and hematoxylin counterstaining (Roche Diagnostics, Indianapolis, IN). A positive control section was included with every batch. The slides were examined according to the NVSL standards, single slides were analyzed and sections of examined lymph node included at least six follicles to allow for a diagnosis of negative in nodes without IHC labeling. Tissues were classified as positive if they had characteristic bright red, granular immunolabeling in the follicular centers.

Real-Time Quaking-Induced Conversion Procedure

For RT-QuIC, RLNs were analyzed either unblinded (n = 698) or in a blinded manner (n = 602). Lymph nodes were trimmed and homogenized in a tube containing beads (ceramic bead and lysing matrix A, MP Biomedicals, Irvine, CA) and 1X PBS with 0.1% sodium dodecyl sulfate at 10% weight per volume using a FastPrep® (MP Biomedicals, Irvine, CA) bead-beater homogenizer (five cycles of 45 s at 6.5 m/s) with care to avoid any cross contamination among tissue homogenates. The homogenized samples were then immediately used or stored at−80°C until processed. RT-QuIC assays were performed as previously described (33) with a few modifications. Briefly, the RT-QuIC reactions were set up in 384-well clear bottom optic plates (Nunc) and consisted of 49 µl of RT-QuIC buffer (0.1 mg/ml truncated Syrian hamster recombinant prion protein (CWD Evolution, San Diego, CA), 320 mM NaCl, 20 mM Na_2HPO_4, 1mM EDTA, and 10 µM Thioflavin T) and 1 µl of sample. Positive control (ELISA, IHC and RT-QuIC positive sample), negative control (ELISA, IHC and RT-QuIC negative sample) and samples consisted of serial dilutions (10^{-3} and 10^{-4}) of a 10% lymph node homogenate and were run in triplicate if 10^{-3} and 10^{-4} dilutions were used or quadruplicate if only dilution 10^{-3} was being tested. The 384-well plates were placed in a BMG Fluostar Omega plate reader with settings of 50 °C for 40 h with cycles of 1 min rest and 1 min shake (700 rpm, double orbital). Thioflavin T fluorescence measurements were taken every 15 min at a gain of 1,600 and excitation of 450 nm and emission of 480 nm. Data were processed using Mars Analytical Software (BMG Labtech, Cary, NC). Reactions were considered positive if fluorescence values exceeded the cycle threshold (average of the ten first readings plus 10 standard deviations). The time to threshold was defined as the time at which a sample fluorescence emission crossed the cycle threshold. Following the first analyses, every sample which showed partial positive reactions (1 to 3 out of 4 replicates) or did not have the same result as the ELISA test was reanalyzed. A sample was considered positive when at least half of total replicates surpassed the threshold.

Statistical Analysis

Statistical analysis (mean, standard error of the mean (S.E.M.), *kappa* value and the percent agreement) were calculated using Microsoft Excel (Microsoft Inc.).

TABLE 1 | Number of positive, negative, not available (N/A), and total of RLNs tested by each assay.

	Positive	Negative	N/A	Total
ELISA	184	1,116	0	1,300
RT-QuIC	178	1,122	0	1,300
IHC	176	195	1	372*

*IHC was performed in all RT-QuIC-positive and ELISA-positive samples, except for one sample that we did not have enough tissue to be analyzed by IHC (N/A = 1), and 188 randomly selected negative RLNs. N/A, not available material to be analyzed; *IHC was performed in RT/QuIC-positive and ELISA-positive samples (n = 184) and in 188 randomly selected negative samples.*

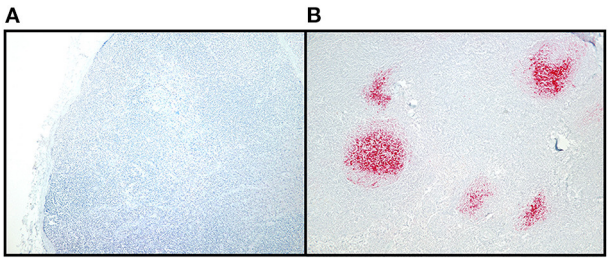


FIGURE 2 | CWD detection in WTD RLN by IHC. Sections of examined lymph node included at least six follicles to allow for a diagnosis of negative in nodes without IHC labeling. Tissues were classified as positive if they had characteristic bright red, granular immunolabeling in the follicular centers. **(A)** IHC staining of a CWD negative RLN, at 10X magnification. **(B)** IHC staining of a positive RLN at 10X magnification.

RESULTS

RT-QuIC Results Are Comparable to ELISA and IHC

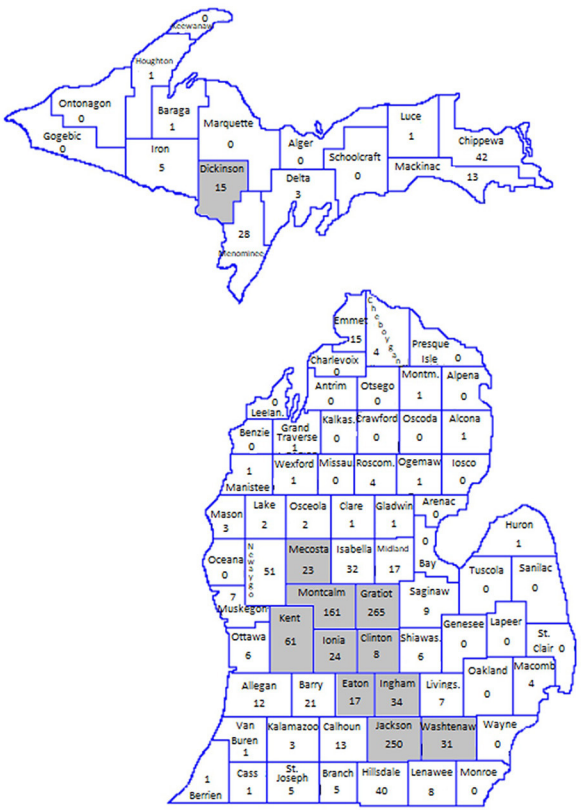
A comparison between currently recognized diagnostic methods for CWD, such as IHC and ELISA vs. new amplification assays, such as RT-QuIC is an important step to provide insight into the sensitivity and specificity of gold standard diagnosis and on the validation of new tools.

Of the 1,300 RLNs tested, 184 (14.2%) were CWD positive by ELISA and 178 (13.7%) by RT-QuIC. Of the 184 ELISA and/or RT-QuIC positive samples, 176 were also IHC positive (**Table 1**). IHC positive lymph nodes presented characteristic granular immunolabelling in the follicular centers (bright red staining) (**Figure 2**). There was a total of seven samples out of the 1,300 RLNs tested (0.5%) where the results for IHC, ELISA, and RT-QuIC did not match. All seven samples were IHC negative and ELISA positive. Six out of the seven discordant samples presented negative RT-QuIC results, which matched the IHC outcomes. One sample was positive by RT-QuIC and ELISA and negative by IHC (**Table 2**). The percent agreement and *kappa* value between RT-QuIC and ELISA were 99.5 % and 0.98, respectively and between RT-QuIC and IHC were 99.7 % and 0.99, respectively. The percent agreement between ELISA and IHC was 98.1% and the *kappa* value 0.96.

TABLE 2 | Samples with discordant results between ELISA, IHC, and RT-QuIC.

Sample ID	Sex	Age (yo)	Location	Average ELISA OD	ELISA result	IHC result	RT-QuIC result
454295	M	1.5	Clinton	0.513	Positive	Negative	Negative
582500	F	1	Macomb	0.167	Positive	Negative	Negative
583708	F	0	Mecosta	0.644	Positive	Negative	Negative
576488	F	4	Macomb	0.211	Positive	Negative	Negative
685995	F	2	Washtenaw	0.261	Positive	Negative	Negative
521225	M	1	Clinton	0.065	Positive	Negative	Negative
575795	M	1	Macomb	3.328	Positive	Negative	Positive

ELISA positive, sample with average value > 0.035 plus the average of the negative control. IHC was performed at the MSU-VDL and NVSL and a sample was considered IHC positive if a positive result was reported by at least one of those laboratories. RT-QuIC threshold was calculated by the average fluorescence of the ten first readings plus 10 standard deviations. A sample was considered RT-QuIC positive when at least half of the total replicates surpassed the threshold. Yo, years old; OD, optical density.



County	Number of positives/ Number of samples tested		
	IHC	RT-QuIC	ELISA
Clinton	2/3	2/8	4/8
Dickinson	1/4	1/15	1/15
Eaton	1/7	1/17	1/17
Gratiot	6/10	6/265	6/265
Ingham	2/14	2/34	2/34
Ionia	3/11	3/24	3/24
Jackson	25/54	25/250	25/250
Kent	31/38	31/61	31/61
Mecosta	0/8	0/23	1/23
Montcalm	105/121	107/161	109/161
Washtenaw	0/2	0/31	1/31
Undetermined	1†	0	0
Total	176/272*	178/889*	184/889*

†Undetermined: sample with not enough material to be analyzed by IHC
*Denominators values varied depending on the number of samples analyzed by each assay

FIGURE 3 | Amount and location of positive samples in Michigan. Map shows the total number of RLN samples tested in each County. Gray represents the Counties with CWD positive results. The table shows the number of positive samples/number of samples tested by County and by each technique used. RT-QuIC and ELISA were performed in 1,300 WTD RLNs. IHC was performed in all RT/QuIC-positive and ELISA-positive samples ($n = 184$) and in 188 randomly selected negative samples.

Characteristics of the Positive CWD Cases

All, except one CWD positive RLNs analyzed were from ten Counties geographically located in the West Michigan region of the Lower Peninsula (**Figure 3**). Montcalm County alone accounted for more than half of the total of positive cases detected in this work (between 109 and 107 positives depending on the test performed). The other positive samples were concentrated in surrounding Counties: Clinton (2 positives by RT-QuIC and IHC and 4 by ELISA), Eaton (1 positive by all three assays), Gratiot (6 positives by all three assays), Ingham (2 positives by all three assays), Ionia (3 positives by all three assays), Jackson (25 positives by all three assays), Kent (31 positives by all three assays), Mecosta (1 positive by ELISA), Washtenaw (1 positive by ELISA) (**Figure 3**). One positive sample (by all three assays) was originated from Dickinson County in the Michigan Upper Peninsula (**Figure 3**).

TABLE 3 | Positive PrP^{CWD} RLNs by sex.

	Total of positive (% of positive)*			Total of samples tested		
Sex	**IHC***	**RT-QuIC**	**ELISA**	**IHC**	**RT-QuIC**	**ELISA**
Female	80	81 (9.9%)	85 (10.4%)	154	819	819
Male	95	96 (20.4%)	98 (20.8%)	216	470	470
Unknown	1	1 (9.1%)	1 (9.1%)	2	11	11
Total	176	178 (13.7)	184 (14.1%)	372	1,300	1,300

*The table presents the total and percentage of positive RLNs and the total of samples tested by sex and assay performed. It is important to note that the percentage of positive samples by sex was not calculated for IHC since only RT/QuIC-positive and ELISA-positive samples (n = 184) and 188 randomly selected RLNs were tested by this assay. *Percentage of positive samples by sex was not calculated for IHC since the assay was only performed in RT/QuIC-positive and ELISA-positive samples (n = 184) and 188 randomly selected negative samples.*

TABLE 4 | Positive PrP^{CWD} RLNs by age category.

	Total of positive (% of positive)*			Total of samples tested		
Years old	**IHC***	**RT-QuIC**	**ELISA**	**IHC**	**RT-QuIC**	**ELISA**
0 to 1	38	40 (8.0%)	43 (8.6%)	118	501	501
> 1 to 2	68	68 (20.1%)	70 (20.6%)	125	339	339
> 2 to 3	37	37 (16.4%)	37 (16.4%)	73	225	225
> 3 to 4	26	26 (21.7%)	27 (22.5%)	38	120	120
> 4 to 5	4	4 (7.0%)	4 (7.0%)	9	57	57
> 5	3	3 (5.8%)	3 (5.8%)	6	52	52
Unknown	0	0	0	3	6	6
Total	176	178 (13.7%)	184 (14.1%)	372	1,300	1,300

*The table presents the total and percentage of positive RLNs and the total of samples tested by age category and assay performed. Age classes were determined based on tooth wear by experienced staff at the Michigan Department of Natural Resources-Wildlife Disease Laboratory. It is important to note that the percentage of positive samples by age category was not calculated for IHC since only RT/QuIC-positive and ELISA-positive samples (n = 184) and 188 randomly selected negative RLNs were tested by this assay. *Percentage of positive samples by age category was not calculated for IHC since the assay was only performed in RT/QuIC-positive and ELISA-positive samples (n = 184) and 188 randomly selected negative samples.*

Of the 1,300 samples analyzed, 20.2% of males were CWD positive in contrast with 9.9% of the females (**Table 3**). More than 3 years old (yo) to 4 yo animals had the highest percentage of positive cases (21.7%), followed by more than 1 to 2 yo animals (20.1%). The percentage of more than 2 to 3 yo positive CWD deer was 16.4%. The percentage of CWD positive cases was smaller in fawns (from 0 to 1 yo animals) (7.8%) and in older animals (more than 4 to 5 yo with 7% and WTD more than 5 yo with 5.8%) (**Table 4**).

PrP^{CWD} Detected in Prefemoral, Prescapular and Popliteal Deer Lymph Nodes

RLNs play an important role as a portal of entry for CWD in early accumulation and propagation of PrP^{CWD} in deer (34). Other early sites of prion accumulation are tonsils, Peyer's patches, and ileocaecal lymph nodes, followed by progressive involvement of central and peripheral nervous tissues (34, 35). However, the exact progression of the PrP^{CWD} deposition in different anatomic locations and the disease stage at which it occurs in peripheral lymph nodes and surrounding tissues is not well understood. This is especially important, since, even if there is no published evidence for CWD transmission to human-like primates at this time, the CWD species barrier between cervids and humans may not be fixed (36, 37). The risk for interspecies transmission through the consumption of CWD-contaminated meat may increase as CWD continues to spread and adapt (38).

A set of RLNs, prescapular lymph nodes, prefemoral lymph nodes and popliteal lymph nodes (**Figure 1**) from eight WTD were tested by ELISA, IHC and RT-QuIC. Animals that presented positive CWD results in the RLNs by ELISA, IHC, and RT-QuIC ($n = 7$), also presented PrP^{CWD} in all the other lymph nodes when tested by IHC and RT-QuIC (**Table 5**). The ELISA, on the other hand, did not detect PrP^{CWD} in prefemoral and popliteal lymph nodes from one positive deer (**Table 5**), but the results of prescapular lymph node and RLN from the same animal tested positive. All lymph nodes tested from CWD-negative control deer were negative by ELISA, IHC, and RT-QuIC.

DISCUSSION

CWD was first identified more than 50 years ago in a captive mule deer at Colorado State University (Fort Collins, Colorado). Since its discovery, the disease has been reported across North America, in South Korea, and in Europe (1–5). In Michigan, CWD was first described in a deer farm in Kent County in 2008. Since 2015, when the first free-ranging CWD-positive deer was identified in Michigan, the disease has been reported in

TABLE 5 | PrP^{CWD} detection on RLNs, prescapular lymph nodes, prefemoral lymph nodes, and popliteal lymph nodes of WTD by ELISA, IHC, and RT-QuIC.

				Retropharyngeal LN				Prescapular LN				Prefemoral LN				Popliteal LN			
Sample	Sex	Age	Location	ELISA Avg. OD	ELISA Result	IHC Result	RT-QuIC Result	RT-QuIC Avg. time to Threshold	ELISA Avg. OD	ELISA Result	IHC Result	RT-QuIC Result	RT-QuIC Avg. time to Threshold	ELISA Avg. OD	ELISA Result	IHC Result	RT-QuIC Result	RT-QuIC Avg. time to Threshold	ELISA Avg. OD	ELISA Result	IHC Result	RT-QuIC Result	RT-QuIC Avg. time to Threshold
702671	M	1.5	Jackson	3.08	positive	positive	positive	10.31	1.275	positive	positive	positive	18.12	N/A	N/A	positive	positive	8.87	0.696	positive	positive	positive	15.4
702880	F	0	Jackson	4.14	positive	positive	positive	8.12	3.555	positive	positive	positive	8.73	4.118	positive	positive	positive	7.23	3.914	positive	positive	positive	7.93
675730	F	4	Jackson	2.24	positive	positive	positive	7.56	2.502	positive	positive	positive	6.13	N/A	N/A	N/A	N/A	N/A	2.29	positive	positive	positive	8.07
674269	F	2	Jackson	2.94	positive	positive	positive	6.01	4.105	positive	positive	positive	7.2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	positive	positive	6.84
674291	F	5	Jackson	3.48	positive	positive	positive	6.56	4.125	positive	positive	positive	6.04	4.044	positive	positive	positive	6.71	4.154	positive	positive	positive	5.83
674292	F	0	Jackson	0.24	positive	positive	positive	7.03	0.227	positive	positive	positive	6.3	0.008	negative	positive	positive	11.68	0.01	negative	positive	positive	17.56
674780	F	3	Jackson	3.782	positive	positive	positive	9.13	1.766	positive	positive	positive	11.3	3.368	positive	positive	positive	11.15	4.079	positive	positive	positive	10.65
420830	F	12	Hillsdale	0.011	negative	negative	negative	N/A	0.007	negative	negative	negative	N/A	0.01	negative	negative	negative	N/A	0.01	negative	negative	negative	N/A

ELISA positive, sample with average value > 0.035 plus the average of the negative control. RT-QuIC threshold was calculated by the average fluorescence of the ten first readings plus 10 standard deviations. The time to threshold was defined as the time at which a sample fluorescence emission crossed the cycle threshold. A sample was considered RT-QuIC positive when at least half of the total replicates surpassed the threshold. N/A, not available.

several counties in the Lower Peninsula. In October 2018, the first CWD-positive deer was found in the Upper Peninsula in Dickinson County (**Figure 3**)[1] (39). As the endemic areas have expanded, so has the need for rapid, sensitive, and cost-effective diagnostic tests. The current gold standard for confirmatory CWD testing in WTD is IHC of the medial RLN; however, ELISA is a high-throughput test often used to screen samples prior to IHC (31, 40). These antibody-based prion detection tests have been very helpful in providing an understanding of pathogenesis, transmission, and geographic distribution of CWD and other prion diseases over the past few decades. However, there are many concerns about their sensitivity especially for animals in early stages of the disease to be effective in robust surveillance programs (41–43). The development of *in vitro* amplification assays, as protein misfolding cyclic amplification and RT-QuIC have helped to enhance the sensitivity of CWD prion detection in the last few years. The RT-QuIC assay has been reported to amplify PrP^{CWD} seeds present in brain dilutions in the femtogram range (44, 45) and has been used to detect PrP^{CWD} in a variety of anatomic sites, body fluids, and excreta (13, 22–30), but the technique has yet to be approved for official CWD surveillance.

In this work, we first evaluated the efficacy of the RT-QuIC assay in detecting CWD prions in RLNs of WTD and compared with the results from IHC and ELISA. We found that our RT-QuIC results were comparable to those of the IHC and ELISA, with only 0.5% (7 samples out of 1,300) of discordant results between the three techniques when testing WTD RLNs (**Table 2**). In previous studies that compared the three assays, 100% of matching results were observed (23, 30). One possible explanation for the observed difference is the fact that the previous studies did not include as many positive WTD RLNs. All the discordant samples in our study were IHC negative but positive by ELISA ($n = 7$). A previous study comparing ELISA and IHC results also detected inconsistent results between the two techniques in 12 out of 101 (11.9%) RLNs tested (46). The same study demonstrated the importance of testing contralateral RLNs by IHC since PrP^{CWD} may not be bilaterally present in those tissues (46). Our failure to confirm ELISA diagnoses with IHC and RT-QuIC are likely either due to ELISA results in discrepant samples were false positives, especially on the samples with low OD ELISA value ($n = 6$) where IHC and RT-QuIC presented matching results (**Table 2**) or due to the sampling methods and protein distribution within the RLN, since the practice of using 1 section of lymph node per animal for IHC may result in false-negative results, especially in early-infection cases (41). RT-QuIC assay only requires sampling between 70 and 100 mg compared with 180–220 mg needed for ELISA, which are preferentially collected from the lymph node cortex to improve accuracy. Nevertheless, one potential caveat of both ELISA and RT-QuIC is that it is likely not possible to be certain that the correct part of the tissue sample was collected and tested. Whereas, IHC examines cross sections of RLN that are 5–7 μm in thickness and include both cortex and medulla. Moreover, the sections collected for IHC contain a

[1]https://www.michigan.gov/dnr

fraction of the tissue sampled by ELISA and RT-QuIC. Therefore, it should be expected that several sections sometimes must be prepared for IHC before positive follicles are observed in early CWD cases (41). Ultimately, we observed that all 176 samples that were positive by IHC were also positive by RT-QuIC. Conversely, no IHC-positive/RT-QuIC-negative samples were identified (**Table 2**). Only one of our discordant results was a RT-QuIC-positive sample (also ELISA-positive) that was negative by IHC at MSU-VDL and NVSL. Previous studies have shown that RT-QuIC positivity precedes and ultimately correlates with PrP^{CWD} deposition observed by IHC, reflecting greater sensitivity of RT-QuIC vs. IHC especially for early CWD detection (47, 48). However, our sample is most likely from an animal with established CWD disease, since it presented a high mean OD value (3.328) on ELISA (**Table 2**). The same was observed in another study comparing ELISA and IHC, where a sample with a high OD value was negative by IHC (41). The result may be explained by differences in methodologies or affinity between antiPrP antibody used in the ELISA, recombinant prion protein used in the RT-QuIC, and the monoclonal antibody used for IHC. Another explanation would be differences in the spatial distribution of the PrP^{CWD} in the samples tested by each assay. Taken together, our results show a great correlation of results between the three techniques, with an agreement between RT-QuIC and the other assays > 99.5% and *kappa* value > 0.98, which was greater than the agreement and *kappa* value observed between IHC vs. ELISA (98.1% and 0.96, respectively). Since the spatial distribution of prion protein within the RLN may play a role in whether protein is detected (41), to achieve a higher confidence in test results, it seems prudent to examine multiple sections of the RLNs, as well as both RLNs to confirm or refute CWD infection. Furthermore, more studies are needed to evaluate the efficiency of different recombinant prion protein in samples from different CWD strains.

Similar to what has been reported for scrapie in sheep (49), PrP^{CWD} spreads throughout the body following a general pattern of prion accumulation, characterized by relatively rapid and widespread prion accumulation in the lymphatic tissues, followed by progressive involvement of and lesions in central and peripheral nervous tissues (34, 35, 50). Lastly, involvement of a wider variety of tissues and organs throughout the body, including endocrine system, heart, kidney, lung, and muscle tissues can be observed, albeit in small amounts, as animals become terminally ill (29, 35). Nevertheless, the exact progression of the PrP^{CWD} deposition and what time in the disease progression it happens in peripheral tissues is unclear. Mule deer fawns inoculated orally with CWD showed early accumulation of PrP^{CWD} in Peyer's patches and ileocecal lymph nodes, as well as the RLNs, as early as 42 days post infection (34). However, the proportion of positive follicles was higher in RLNs than in Peyer's patches and ileocecal lymph nodes from 42 to 80 days post infection (34), suggesting that RLNs may play an important role as a portal of entry for CWD in early propagation and accumulation of PrP^{CWD} in deer. Another study observed PrP^{CWD} deposits in tonsils and lymph nodes prior to detectable accumulation in intestinal lymphoid aggregates at 90 days post infection (35). Furthermore, a study shown that peripheral accumulation and the excretion of the infective prion protein can occur at the same time as the peripheral lymphoid accumulation (51). Finally, in very early cases of CWD, PrP^{CWD} deposition is limited and since RLNs and medulla oblongata at the level of the obex are early sites of prion accumulation, they are considered gold standard tissues for post-mortem CWD detection (35).

In this study, in addition to analyzing the performance of the RT-QuIC assay compared with ELISA and IHC in RLNs, we also tested a broad set of WTD lymph nodes from different parts of the body (**Figure 1**). We found that in addition to the RLN, PrP^{CWD} was also present in prescapular lymph nodes, prefemoral lymph nodes, and popliteal lymph nodes of all CWD positive WTD tested here (**Table 5**). Our results are concordant with previous work reporting that CWD prions are distributed across lymphoid tissues from deer orally infected with CWD (34, 35, 52). RT-QuIC and ELISA were able to detect PrP^{CWD} in all the lymph nodes tested from the CWD-positive animals. On the other hand, ELISA was negative in samples from prefemoral lymph node and popliteal lymph node from one of the CWD-positive WTD. The same animal presented positive ELISA results when RLN and prescapular lymph nodes were tested (**Table 5**). It is interesting to note that this animal presented low OD ELISA values of 0.24 and 0.227, for RLN and prescapular lymph node, respectively (plate-to-plate cut-off ELISA OD values ranged from 0.038 and 0.08, data not shown), which is indicative of early CWD infection. It is known that after entering the body, either orally and/or intranasally, CWD prions typically accumulate within the lymphatic tissues of the head and neck, such as the RLNs, before spreading from the head/neck lymphoid tissues to the rest of the body (34, 35, 50). The RT-QuIC results for the same animal also suggest low accumulation of PrP^{CWD} in the prefemoral lymph node and popliteal lymph node, which were detected positive after 11 and 17 h, respectively, compared to 7 h for RLN and 6 h for prescapular lymph node. Our results suggest that RT-QuIC may have been more sensitive than ELISA to detect PrP^{CWD} accumulation in lymph nodes other than the RLN in animals in early stages of the disease. Although additional research, including more lymphoid tissues from different parts of the body, is needed to determine the full extent to which CWD prions occur within specific lymph nodes and surrounding tissues of infected animals.

Deer behave differently among sex and age classes, and as a result, CWD prevalence can differ among different sex and age groups. Most likely, the sex and age effects are mainly driven by differences in pathogen exposure (53) and therefore, strongly depend on the social organization or behavior of a given species. In our study, we observed a two-fold higher CWD prevalence in males (20.2%) than in females (9.9%) WTD (**Table 3**). These results are in accordance with previous work in both mule deer and WTD (54–56). Female deer are known to form matrilineal groups with stable home ranges where they consistently interact with the same members of their matrilineal groups (54, 57). On the other hand, males are hypothesized to have higher CWD prevalence due to behavioral traits related to serial polygamy, and longer movement distances, increasing the overall likelihood of visiting an infected group or spreading the disease to new

groups (57, 58). On the other hand, a previous study observed higher CWD prevalence in females when compared with males, suggesting that in some more heavily CWD affected areas, environmental transmission in late epidemic stages may erode the sex-specific infections in more concentrated populations (59). As previously reported (54, 56, 60), we also found that CWD prevalence is very low in fawns (7.8% CWD prevalence in WTD < 1 year old) when compared with other age groups. This observation can be explained by the pattern of infection across age classes. CWD likely arises due to differences in the prion exposure period since birth and the long incubation period of the infection before it can be detected by standard diagnostic tests (61). We observed that CWD prevalence increases in yearlings (between 1 and 2 yo; 20.1%), declining slightly in the third year of life (between 2 and 3 yo; 16.4%), and returning to increase with a peak in the fourth year of life (between 3 and 4 yo; 21.7%) (**Table 4**). After the fourth year of life (more than 4 yo), CWD prevalence declines steadily. In agreement with our results, it has been previously reported that prevalence levels often continue to increase moderately with age in the adult stage, where greater movement and interactive behaviors lead to higher infection risk (60, 62). Furthermore, a decline in CWD infection among the oldest deer has also been previously reported in both WTD and mule deer (59, 62) that can result from decreased movement and interaction behaviors leading to a lower force of infection and disease-induced mortality removing older individuals from the population.

Taken together, we show evidence that the RT-QuIC assay is comparable to ELISA and IHC for CWD detection and could be used as a new tool for the detection of CWD prions. In this study, RT-QuIC and IHC (which is the gold standard technique for CWD diagnostic) results showed better correlation than with ELISA. One of the advantages of the RT-QuIC is that it can easily scaled up when compared to IHC and ELISA, since it can be performed in 384-well plates, which is amenable to CWD surveillance programs across the globe. Another advantage of the RT-QuIC is that the final product is not infectious. When compared to IHC, RT-QuIC is less expensive and less laborious. RT-QuIC can also portends IHC positivity in signifying early CWD infection before it can be detected by IHC (47, 48). Moreover, RT-QuIC results can be visualized while the assay is running, and a positive sample can be detected as soon as the fluorescence values start to increase. Furthermore, RT-QuIC can also be used in a variety of tissues, body fluids, and excreta from animals at clinical, and sometimes at subclinical stages of the disease (13, 22–29). Finally, it can also provide a range of information that may be used for the assessment of tissue burden and environmental contamination, which will allow for more detailed studies into disease epidemiology and pathogenesis (25, 63, 64). Our findings also suggest that CWD prions occur throughout an array of WTD lymph nodes, but further studies focusing on larger sample sizes are necessary to understand the extent of this distribution not only in lymphoid tissues, but also in surrounding organs and tissues. Continued improvements in RT-QuIC methodologies, especially for antemortem CWD diagnosis and multi-laboratory validation on blinded sample panels are ongoing and will aid in defining an algorithm for RT-QuIC application in prion diagnostics and routine CWD surveillance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CH, KS, SB, and SS designed the study. CH, JD, NG, and MK performed the experiments. CH and JD analyzed the data. CH and SS wrote the manuscript. All authors read and approved the final manuscript.

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RESEARCH PAPER

Management of chronic wasting disease in ranched elk: conclusions from a longitudinal three-year study

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ABSTRACT

Chronic wasting disease is a fatal, horizontally transmissible prion disease of cervid species that has been reported in free-ranging and farmed animals in North America, Scandinavia, and Korea. Like other prion diseases, CWD susceptibility is partly dependent on the sequence of the prion protein encoded by the host's *PRNP* gene; it is unknown if variations in *PRNP* have any meaningful effects on other aspects of health. Conventional diagnosis of CWD relies on ELISA or IHC testing of samples collected post-mortem, with recent efforts focused on antemortem testing approaches. We report on the conclusions of a study evaluating the role of antemortem testing of rectal biopsies collected from over 570 elk in a privately managed herd, and the results of both an amplification assay (RT-QuIC) and conventional IHC among animals with a several *PRNP* genotypes. Links between *PRNP* genotype and potential markers of evolutionary fitness, including pregnancy rates, body condition, and annual return rates were also examined. We found that the RT-QuIC assay identified significantly more CWD positive animals than conventional IHC across the course of the study, and was less affected by factors known to influence IHC sensitivity – including follicle count and PRNP genotype. We also found that several evolutionary markers of fitness were not adversely correlated with specific *PRNP* genotypes. While the financial burden of the disease in this herd was ultimately unsustainable for the herd owners, our scientific findings and the hurdles encountered will assist future CWD management strategies in both wild and farmed elk and deer.

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Introduction

Chronic wasting disease (CWD) is a naturally occurring, progressive and ultimately fatal prion disease of cervids, including mule deer (*Odocoileus hemionus*), whitetail deer (*Odocoileus virginianus*), Rocky Mountain elk and red deer (*Cervus elaphus* sspp.), reindeer (*Rangifer tarandus*) and moose (*Alces alces*) [1–3]. The disease has been identified in free-ranging and farmed cervids in 26 US states and 5 countries outside of the United States – most recently with novel disease foci reported across northern portions of the Scandinavian Peninsula [4–6]. New foci of the disease have been uncovered at the rate of roughly one state per year since its initial discovery outside of the original endemic zone of northern Colorado and southern Wyoming in the late 1990s.

The management of CWD in wild deer and elk herds has proven difficult, if not impossible, since being found in free-ranging mule deer and elk four decades ago [7]. Currently, the state of New York is singular in its effective surveillance and eradication of a small focus of CWD in whitetail deer, however the disease is fairly well established in wild cervid populations in neighbouring Pennsylvania and has recently been reported in captive facilities in neighbouring Ohio and Quebec [8–10]. In 2018, Norway attempted to significantly reduce the population size of reindeer in one area where CWD was discovered [11], however the discovery of the disease in neighbouring Finland and Sweden suggests the endemic area may be larger than initially believed.

Discovery of CWD on privately-owned cervid farms results in immediate quarantine of the premises and almost inevitably herd depopulation, with farmers compensated by federal and/or state indemnity funds [12]. Very rarely, property owners forgo indemnity and depopulation, and attempt to manage their herds under

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Supplemental data for this article can be accessed here.

a strict quarantine [13]. These unique situations provide for the limited development of small-scale management strategies in controlled populations that could someday be applied to the management of larger, free-ranging herds. This manuscript describes one such property, a herd of over 570 elk maintained on 3500 acres of fenced habitat in northwestern Colorado.

We began the study hopeful that CWD could be effectively managed using a combination of early detection using rectal biopsies and the culling of CWD-positive animals, though ultimately the financial strain of managing a large CWD positive elk herd under near free-ranging conditions became too difficult for the herd owners to bare. During the second half of the project, we attempted to identify cows with specific alleles for the elk prion gene, *PRNP*, known to correlate with lower CWD susceptibility [14], including both 132LL homozygous and 132ML heterozygous animals, and separate them from the rest of the herd. Our expectations were that these cows would be selectively bred to increase the frequency of less susceptible alleles in the herd at large, however they remained unbred as management objectives evolved into a depopulation effort. Taking advantage of data available, we explored the relationships between CWD, elk genotype, and fitness, and investigated correlations between both CWD status and *PRNP* genotype with pregnancy, body condition score (BCS) and calf survival. Throughout the study, we continued to compare the performance of a prion amplification assay, RT-QuIC, with conventional immunohistochemistry in the antemortem detection of CWD, ultimately correlating our findings to post-mortem testing and survival.

Despite fluctuations in management objectives over the course of the study, we report a number of important findings. First, we found that RT-QuIC assay was significantly more sensitive than conventional IHC in the antemortem detection of CWD positive elk in recto-anal mucosa-associated lymphoid tissue (RAMALT), by a factor of nearly 50%. As an antemortem sample in elk, however, RAMALT remained clearly imperfect compared to post-mortem testing of the brainstem and retropharyngeal lymph nodes. Second, we found that CWD negative elk were 3.6 times more likely to survive year over year than those in which CWD was detected antemortem. Third, we report that neither CWD status nor *PRNP* genotype correlated with lower pregnancy rates in elk, and that *PRNP* genotype did not have an obvious effect on calf survival in the first and second year of life. Finally, while no correlation was found between body condition score and either sex or *PRNP* genotype, animals testing positive for CWD antemortem were found to be in generally poorer body condition than those testing negative antemortem.

Over the course of this multi-year longitudinal study, we learned that – as is frequently observed with CWD management in wild populations – the involvement and full cooperation of herd owners (in the case of wild cervids, the public at large) is critical for even the slightest chance of managing the disease. The demise of this particular herd seemed almost inevitable, however, regardless of our management directives. Despite this initial failure, our hope is that this primary effort of managing CWD in farmed elk may serve as a learning tool for future management efforts for this devastating disease in either privately-owned or wild herds.

Materials and methods

Ethics statement

All animals in this study were handled humanely in accordance with Midwestern University's Animal Care and Use Committee, approval #2814. Animals selected for euthanasia were humanely euthanized in accordance with guidelines issued by the American Veterinary Medical Association.

Study area and population

Details on the study population and property have been reported previously [13]. Briefly, the study was conducted in a 3500-acre (14km^2) fenced-in area of private land in Colorado with features similar to other areas of Colorado with endemic CWD. Chronic wasting disease was first reported on the property in 2004, and prevalence rose steadily over the ensuing years. In the first year of this study (2016), prevalence was approximately 15% in adult elk based on antemortem and post-mortem testing (where available). Prevalence in mule deer and elk outside the fence is unknown, although it is presumed to be greater than 5-10% [15,16]. Future proposed testing requirements may provide more insight into current CWD prevalence in the area [17]. The herd initially consisted of over 450 animals in the first year of the project, declining to 400 and eventually 150 in the second and third years of the project (2017 and 2018), respectively. The decline was a combined result of depopulation efforts targeting CWD-positive animals, CWD-associated and unassociated animal deaths, and heavy hunting pressure as the herd was passively depopulated in the second and third years of the study. Animals were handled once yearly in the late winter, as they were run through a modern handling facility for inventory, sample collection, and routine medical treatments. Animals were identified using ear tags, RFID chips, and tattoos. In the winter of 2017, twelve 132LL and thirteen 132ML cow elk were separated out into a 100-acre pasture to be bred to bull elk with 132LL alleles; those cows were never bred and remained fallow in 2018.

Study design

Details on the study design have been reported previously [13]. In short, a range of empirical data and clinical samples were collected from both adult and calf elk during the annual winter inventory. Empirical data included body condition scoring on all animals and pregnancy evaluation of adult cows over two years of age; both data sets were collected by an experienced large animal veterinarian. Clinical samples collected from adult animals included blood, faeces and rectal biopsies as described below. In calves less than one year of age, only blood samples were collected for genetic analysis. Adult cows were held in a separate pen after sampling to allow for the targeted culling of CWD positive individuals. Adult bulls were turned out into a 500-acre bull pen regardless of CWD status, and all calves were released onto a 500-acre spring pasture. Cows positive for CWD were humanely euthanized with a range of samples collected during necropsy, including conventional diagnostic samples. All CWD negative cows were released onto the spring pasture with the calves except for the aforementioned 132ML and 132LL cows.

Antemortem and post-mortem sampling and data collection

During sample collection, animals were restrained using a conventional large animal squeeze chute. Blood was collected from the jugular vein and placed into a tube containing ethylenediaminetetraacetic acid tetrasodium salt (EDTA) preservative. Faeces were collected using manual evacuation, with several pellets placed in storage bags for ancillary testing [18]. Rectal biopsies were collected using sterile, single use instruments by removing a $2cm^2$ piece of mucosa from the wall of the rectum, approximately 2cm proximal to the mucocutaneous junction of the anus as described previously [19,20]. A $0.5cm^2$ subsection of this biopsy was placed into a 1.5ml microcentrifuge tube and frozen for RT-QuIC analysis, with the remainder placed into a histology cassette and preserved in 10% neutral buffered formalin (NBF) for IHC analysis.

Body condition scoring was conducted by both visual analysis and palpation of the ribs, spine, hip bones, rump, tail head and belly of the animals according to published guidelines [21]. Scores ranged from 1 to 5, though observed scores rarely exceeded 3. Pregnancy was assessed by rectal palpation, with animals typically in the late second trimester of pregnancy.

Post-Mortem samples were collected immediately after euthanasia, and where available from animals that died in the field (e.g. those hunted or dying of natural causes). At a minimum, the obex region of the brainstem, the medial retropharyngeal lymph nodes, and tonsil were collected, when available, and stored in 10% NBF for post-mortem assessment of CWD status.

Immunohistochemistry (IHC)

Rectal biopsies and post-mortem samples were evaluated for PrP^{res}-specific immunostaining as described previously, blindly and without information on the index test (RT-QuIC) results [13]. Briefly, IHC staining for PrP^{res} was performed using the primary antibody Anti-prion 99 (Ventana Medical Systems, Tucson, AZ) and counterstained with haematoxylin. Positive and negative controls were included in each analysis. Biopsies were considered positive if at least one follicle exhibited PrP^{res}-specific staining. In cases where biopsies had five or fewer follicles, samples were classified as 'insufficient follicles' unless CWD-specific immunostaining was observed.

Statement of transparency on RT-QuIC testing

Over the course of testing in the third year of study, a single amplification plate was discarded due to equipment malfunction. Cumulatively, 847 patient samples were run on 31 experimental plates over the course of the entire study; of these, three plates were repeated when positive controls failed to amplify, one was repeated due to a single negative control replicate amplifying, and two were repeated due to equipment malfunction.

Real time quaking-induced conversion assay (RT-QuIC)

Rectal biopsy subsections were prepared as 10% homogenates in phosphate-buffered saline (PBS) and analysed for PrP^{res} conversion activity as described previously [6]. Amplification was performed using a truncated form of recombinant Syrian hamster PrP (SHrPrP, residues 90–231) as a conversion substrate. The details on reaction conditions are described elsewhere [14]. Test samples were analysed blindly, without information on the reference test (IHC) results, and were each repeated in triplicate on a single plate. Positive and negative controls (consisting of CWD-positive brain and biopsy homogenates from three known CWD-negative whitetail deer, respectively) were included in each analysis in triplicate. An unspiked 'water negative' negative control was also included in triplicate in each experiment. Reactions were prepared in black 96-well, optical bottom plates, which were then sealed and incubated in a BMG Labtech

Polarstar™ fluorimeter. Amplification parameters and relative fluorescence monitoring are described elsewhere.

Criteria for identification of positive samples was determined *a priori* and was consistent with previous studies in our laboratories [13,19,22,23]. Briefly, a replicate well was considered positive when the relative fluorescence crossed a pre-defined threshold, calculated as ten standard deviations above the mean fluorescence of all sample wells – positive controls, negative controls, and test samples – across amplification cycles 2–8. Positive samples were those which crossed the threshold in ≥2 out of 3 replicates; animals with 1 of 3 replicates positive were considered 'suspects.' Plates were disqualified if positive controls failed to amplify, or if amplification was observed in any of the various negative control replicates.

PRNP analyses

Nucleic acids were extracted from whole blood samples preserved in EDTA using a conventional DNA extraction kit (ThermoFisher, USA). The *PRNP* gene sequence was amplified by conventional PCR and sequenced as previously described [13,22,23]. Sequences were viewed using Geneious software version 10.2 (www.Geneious.com) with specific single nucleotide polymorphisms at position 403 of the elk *PRNP* gene used to classify animals into 132MM, ML, or LL genotypes. Several other nucleotide polymorphisms were observed, though not considered for the present analysis.

Data analysis

Statistical analysis of several components of the data set were analysed using GraphPad Prism 8.0 software. A two-tailed Fisher's exact test was used to compare the number of CWD-positive animals identified by IHC and RT-QuIC, the *PRNP* genotype of those animals which were RT-QuIC positive and either IHC positive or IHC negative, the prevalence of CWD among different *PRNP* genotypes, the return rates of infected and uninfected animals, and the pregnancy status of animals with various CWD and *PRNP* genotypes. A Student's t-test was used to compare the average age of RT-QuIC positive animals which were either IHC positive or IHC negative, as well as the average age of first detection in elk with different *PRNP* alleles.

To analyse the relationships between body condition score, *PRNP* genotype, sex, and CWD status, a three-factor ANOVA analysis with regression was conducted using the Real Statistics Resource Pack software (Release 6.2) [24].

Results

Antemortem detection of CWD in RAMALT by immunohistochemistry and RT-QuIC

In the third year of the study, 141 animals had RAMALT biopsies collected antemortem and tested for CWD, with twenty-nine found to be CWD positive (20.6%). Of those, three were positive by IHC only (one of which was considered suspect by RT-QuIC), twenty were positive by both IHC and RT-QuIC, and six were positive by RT-QuIC only. Two IHC negative elk were considered RT-QuIC suspects, with the remaining 110 animals negative by both assays antemortem. (Table 1 and Figure 1)

Over the course of the three-year study [13], 100 unique animals were identified as CWD positive by antemortem IHC of RAMALT, and of these 71 were confirmed positive by post-mortem IHC; the remaining 29 perished in the field untested. Of the 147 unique individuals identified as RT-QuIC positive across the three-year period, 75 were confirmed positive by post-mortem IHC; the remaining 72 animals perishing in the field. (Figure 1 and Supplementary Figure 1) The large discrepancies between post-mortem sample availability is primarily explained by the management practices, where all cows identified as IHC positive antemortem were euthanized, while the majority of the animals which were IHC negative yet RT-QuIC positive were released. Post-Mortem testing uncovered 38 additional CWD positive animals considered negative by both assays, with 9 testing positive in the RLN only, and 29 testing positive in both obex and RLN several months to a full year after initial antemortem screening. The cumulative number of positive animals identified by antemortem IHC or RT-QuIC, or post-mortem IHC totalled 193 animals. Taken collectively, our findings suggest that the antemortem sensitivity of IHC on RAMALT is roughly 52%, with RT-QuIC identifying significantly more positives with a sensitivity of 76% ($p < 0.01$). Determining specificity of the IHC and RT-QuIC assays given the study design is more problematic, however we can report that no animal testing positive by either assay antemortem was later found to be CWD negative by post-mortem testing. As such, our initial supposition is that the specificity of both assays is 100%.

Factors affecting CWD detection by IHC

To this point, three factors have been implicated to explain why CWD positive elk or deer may not be positive by IHC on antemortem RAMALT testing: low follicle counts, *PRNP* genotype, and stage of infection [19,23,25]. Although we were not able to assess disease stage effectively

Table 1. Summary of antemortem and post-mortem testing by assay and result. Chronic wasting disease infection status in elk was categorized based on immunohistochemistry (IHC) and real time quaking-induced conversion (RT-QuIC) results from rectal biopsies. Where available, post-mortem samples were collected from animals in the second year of the study either following euthanasia or from animals harvested in the field, to confirm their CWD status. Animals from the second year of sampling which were not harvested, and did not return for the third year of the study, were presumed to have died in the field, with no sample available (NSA) for post-mortem testing. Final post-mortem test results from the third year of the study were typically not available.

Study Year Two (2017)							Study Year Three (2018)				
			Post-Mortem Test Result				Antemortem Test Result				
Antemortem Test Result	Sex	Number	CWD(+)	CWD(-)	NSA	Returned	IHC(+) only	IHC(+), RT-QuIC(+)	RT-QuIC(+) only	IHC(-), RT-QuIC Suspect	(IHC(-), RT-QuIC(-)
IHC(+),	Bull	0	0	0	0	0					
RT-QuIC(-)	Cow	2	2	0	0	0					
IHC(+),	Bull	0	0	0	0	0					
RT-QuIC Suspect	Cow	2	2	0	0	0					
IHC(+),	Bull	19	1	0	17	1	0	1	0	0	0
RT-QuIC(+)	Cow	30	29	0	1	0					
IHC(-),	Bull	8	2	0	6	0					
RT-QuIC(+)	Cow	10	1	0	7	2	0	1	1	0	0
IHC(-),	Bull	6	1	1	3	1	0	1	0	0	0
RT-QuIC Suspect	Cow	5	1	1	3	0					
IHC(-),	Bull	81	3	20	15	43	2	7	0	1	33
RT-QuIC(-)	Cow	152	21	70	25	36	0	7	4	1	24
Untested Calves	Bull	37	0	0	13	24	0	0	0	0	24
	Cow	48	1	8	5	34	1	3	1	0	29
Total	Bull	151	7	21	54	69	2	9	0	1	57
	Cow	249	57	79	41	72	1	11	5	1	53

in the present study, we did have data on several other metrics available to examine why RT-QuIC positive samples may be IHC negative. A significantly lower number of follicles was observed in RT-QuIC positive, IHC negative samples compared to RT-QuIC positive, IHC positive samples, and has been reported elsewhere (23.2 vs. 32.3, respectively), though were in both cases generally higher than the five follicle target for greatest sensitivity [26,27]. Over the course of the study, fifteen samples that were RT-QuIC positive had five or fewer follicles on IHC evaluation [25,28], suggesting that high numbers of follicles may not be required for RT-QuIC amplification. Other factors we sought to consider here were animal age and *PRNP* genotype.

While the average age of animals with RT-QuIC positive, IHC negative biopsies across the three-year study period was not significantly different from those with RT-QuIC positive, IHC positive biopsies (5.77 vs. 4.98, $p = 0.1145$), there was a trend towards older, RT-QuIC positive animals being IHC negative. Interestingly, an RT-QuIC positive animal which was IHC negative was more likely to have a 132ML genotype than one which was positive by both assays across the three-year study period ($p = 0.017$). Among the RT-QuIC positive, IHC negative samples, there were twenty-seven 132MM individuals and twenty-nine 132ML animals; among those which were RT-QuIC positive and IHC positive, there were sixty-seven 132MM individuals and thirty-one 132ML animals. Taken together, these latter two points hint that *PRNP* genotype, a variable shown to affect CWD progression in cervids, may have an impact on antemortem testing sensitivity – potentially favouring the RT-QuIC assay.

Prevalence of CWD among elk with different PRNP backgrounds

Between sample collection periods in years two and three, a total of 164 animals were euthanized or hunted and tested for CWD. An additional 95 were lost in the field, thus their post-mortem CWD status could not be determined. Among elk with the 132MM genotype, a total of 35/56 animals (62.5%) were found to be CWD positive by antemortem testing, post-mortem testing, or both, while in animals identified as 132ML heterozygous, 23/98 (23.5%) were found to be CWD positive. Five of twelve 132LL homozygous animals (41.7%) were found to be CWD positive. (Table 2)

In sample collection year three, a total of 141 adult elk were tested antemortem for CWD. Among the 132MM homozygous animals tested, 18/61 (29.5%) were positive by either IHC or RT-QuIC, or both; one animal was considered an RT-QuIC suspect, with the remaining testing negative by both IHC and RT-QuIC. Testing of 132ML heterozygous animals identified 9/65 animals as CWD positive (13.8%); an additional animal was considered suspect by RT-QuIC, and the remaining 55 animals were RT-QuIC negative. Finally, two of fifteen 132LL homozygous animals (13.3%) were found positive by RT-QuIC and/or IHC; the remaining eighteen animals were all negative by both assays. (Table 2)

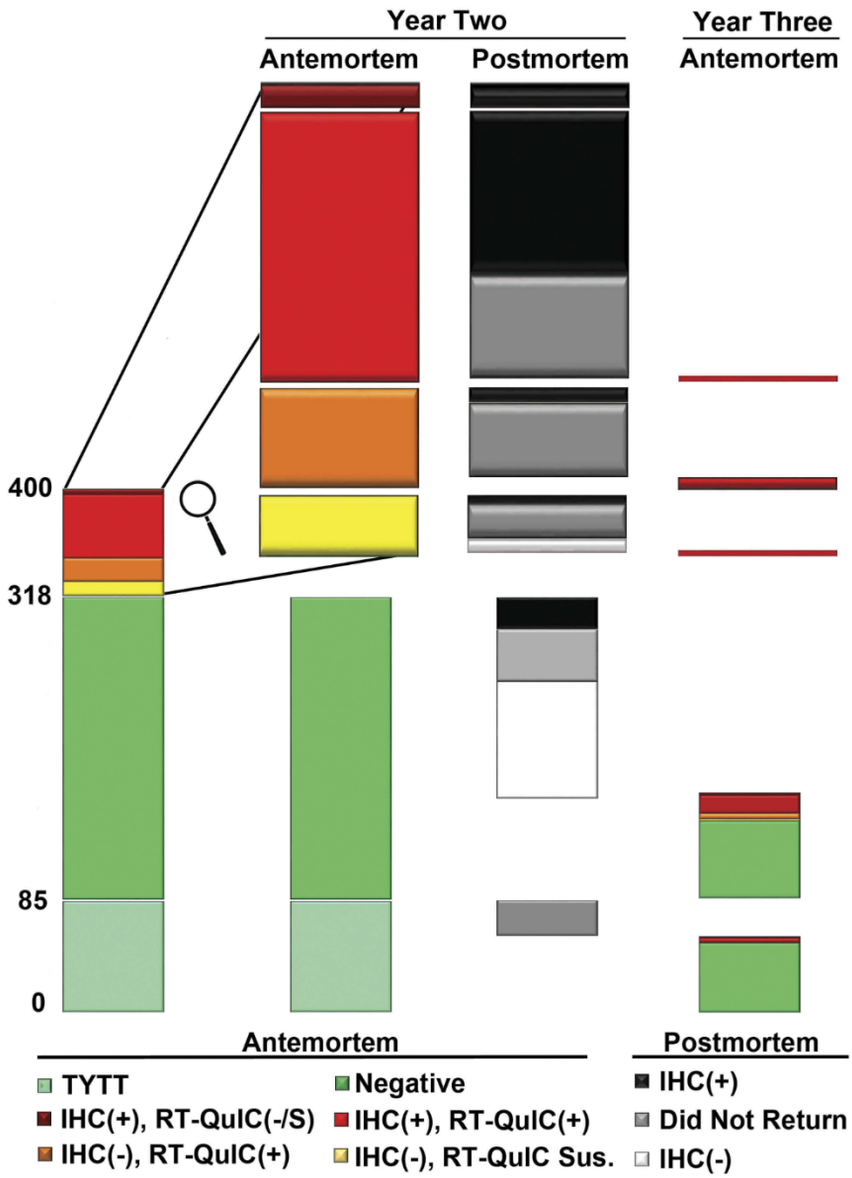

Figure 1. Overview of antemortem testing and post-mortem findings (where available) from study years two and three. Elk were grouped into categories based on testing results, including: (1) "too young to test' (TYTT), (2) negative by both immunohistochemistry (IHC) and real-time quaking induced conversion (RT-QuIC), (3) IHC positive and RT-QuIC negative or suspect, (4) IHC positive and RT-QuIC positive, (5) IHC negative and RT-QuIC positive, and (6) IHC negative and RT-QuIC suspect. No animals testing positive by either antemortem assay were found to be negative post-mortem, with many not returning for sampling the following year.

Table 2. Third year summary of antemortem and post-mortem testing by *PRNP* genotype. Immunohistochemistry (IHC) and real time quaking-induced conversion (RT-QuIC) were used to evaluate rectal biopsies from ranched elk for evidence of chronic wasting disease (CWD). Post-Mortem samples were collected either following euthanasia or when available from animals harvested in the field, to confirm their CWD status. Animals from the second year of sampling which were not harvested, and did not return for the third year of the study, were presumed to have died in the field, with no sample available (NSA) for post-mortem testing. Final post-mortem test results from the third year of the study were typically not available.

		Number Tested			Antemortem test results					Post-Mortem IHC results			
Study Period	132 *PRNP*	Bulls	Cows	Untested Calves	IHC +Only	IHC(+), RT-QuIC(+)	RT-QuIC(+) Only	IHC(-), RT-QuIC Suspect	IHC(-), RT-QuIC(-)	CWD (+)	CWD (-)	NSA	Returned
Year 2	MM	38	74	45	2	36	5	3	66	35	21	40	61
	ML	63	115	30	0	14	11	8	145	23	75	45	68
	LL	13	12	10	0	1	0	0	24	5	7	8	15
Year 3	MM	30	31	1	1	12	5	1	42	17	16	29	0
	ML	29	36	3	1	7	1	1	55	12	21	35	0
	LL	10	5	5	1	1	0	0	13	1	2	17	0

Throughout the study, 565 individual elk, including both adults and calves, were sampled during the course of annual inventory [13]. Among 232 animals identified as 132MM homozygous, 112 were found to be CWD positive either ante- or post-mortem (48.3%). Seventy of 283 animals with the 132ML genotype (24.7%) were found to be CWD positive, while seven of 50 132LL homozygous animals were positive for CWD (14%). Animals homozygous for the 132M allele were nearly twice as likely to be identified as CWD positive compared to their 132ML counterparts (relative risk: 1.95, $p < 0.001$; 95% confidence interval 1.53–2.49). Animals with the 132MM genotype also faced a risk of nearly 3.5 times that of 132LL homozygous genotype (relative risk: 3.45, $p < 0.001$, 95% confidence interval 1.71–6.94). In turn, 132ML heterozygous animals were not significantly more likely to be found CWD positive than 132LL homozygous animals, although there was a trend to indicate they may be (risk ratio: 1.59, $p = 0.19$; 95% confidence interval 0.7815–3.24).

Age at initial CWD detection among elk with different PRNP backgrounds

In an effort to better understand the dynamics of CWD infections in elk with varying *PRNP* backgrounds, we examined the age of initial detection of CWD, by either antemortem or post-mortem testing, in elk with 132MM, 132ML, and 132LL genotypes across all years of the study.

The average age of first detection of CWD in 132MM individuals was 4.98 years, while the average age among 132ML animals was 6.01 years ($p = 0.039$). This difference was most apparent among males, where 132MM bulls were identified as CWD positive at 3.60 years of age, on average, compared to 132ML bulls where the average age of first detection was 4.79 years ($p = 0.01$). The difference between age at first detection in cows, however was not significant (5.76 vs. 6.47 for 132MM and 132ML cows, respectively; $p = 0.27$). The average age of detection in 132LL animals was not significantly different from that of 132ML animals, in either bulls or cows. (Figure 2 and Supplementary Table 1)

Return rate of CWD positive animals

In the 2017 sampling period, 315 animals were tested for CWD antemortem. Of those, 71 tested positive by IHC, RT-QuIC, or both. Thirty-four infected animals (thirty-three cows and one bull) were euthanized and confirmed CWD positive post-mortem, with the remaining thirty-seven animals (Twenty-six bulls and eleven cows) released back onto the property. Of those animals which were released, four were harvested in the fall of 2017 and were found to be CWD positive post-mortem. Three of the remaining thirty (10%) returned for the 2018 sampling period, the remainder were lost in the field and went untested. All three were 132ML heterozygous animals, each positive again on antemortem testing in year three. In contrast, 120 of the 329 animals negative by antemortem testing were harvested in the fall of 2017, with 26 found to be CWD positive (21.7%). Of the remaining 209 animals, 141 returned for the 2018 sampling period (67.5%), with the remaining animals presumed lost in the field, untested. (Table 1 and Figure 1)

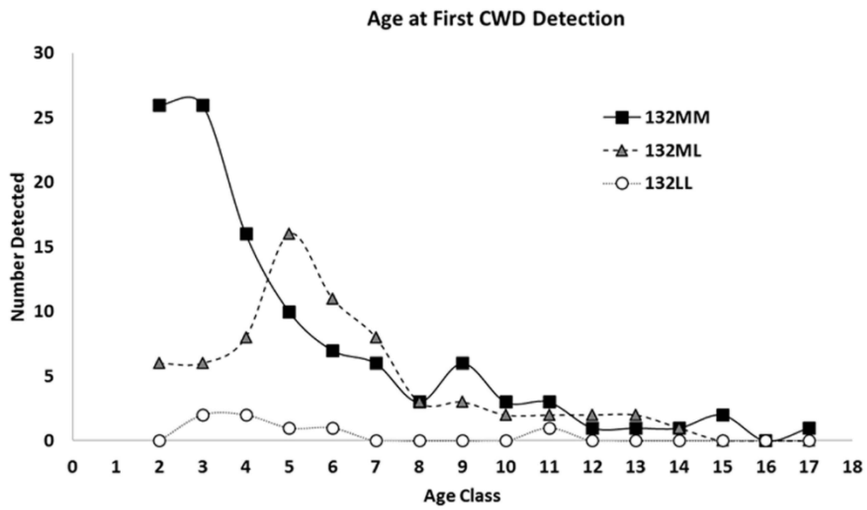

Figure 2. Age of first detection of CWD infection in elk, based on combined antemortem and post-mortem testing data. Antemortem testing combined information collected from both the real-time quaking induced conversion assay (RT-QuIC) and immunohistochemistry (IHC), while post-mortem data was based on IHC testing. Genotypes were confirmed by sequencing of the *PRNP* gene.

Over the course of the entire study, four of forty-nine CWD positive 132MM animals released back onto the property (8.2%) returned for a second year of sampling. Nine of thirty-three 132ML animals positive for CWD returned for a second year of sampling (27%), a return rate that was significantly greater than that of CWD positive 132MM animals (risk ratio: 3.34, p = 0.03). The lone 132LL cow identified during the course of antemortem testing in year two did not return for sampling in year three. Cumulatively, just 13 of 82 animals identified as CWD positive and released onto the property returned the following year (15.9%). These low rates of yearly return are in stark contrast to the cumulative return rate for CWD negative animals. For animals homozygous for the 132M allele, 107/144 returned in year two, and 28/69 returned in year three (63% overall). For 132ML heterozygous animals, 170/221 returned in year two, with 62/181 returning in year three (58% overall). Twenty-five of thirty-five animals homozygous for the 132L allele returned in year two, with fifteen of thirty-five returning in year three (57% overall). Cumulatively, 60% of animals negative for CWD returned the following year – a yearly return rate nearly over 3.5 times that of CWD positive animals (risk ratio: 3.62, p < 0.001; 95% confidence interval 1.96–6.69).

Pregnancy rates in animals with varying PRNP backgrounds and CWD status

Previous studies have found variable influences of *PRNP* polymorphisms on fertility [29,30], while one recent report has evaluated fertility rates of mule deer in an area with endemic CWD and found no relationship between infection status and pregnancy [31]. Over the course of two sampling years, we evaluated late-term pregnancy in 251 cow elk over two years of age to compare the pregnancy rates across various *PRNP* genotypes, as well as to CWD status.

Pregnancy rates did not differ significantly between 132MM and 132ML cows across both years of the study (61% and 68% respectively, $P = 0.27$). Pregnancy rates were significantly higher in 132ML animals compared to 132LL animals (33%, $P < 0.05$), however it should be noted that the separation of 12 adult 132LL cows in the second study year resulted in just five 132LL cows, all 2-year-olds, for inclusion in our pregnancy evaluation in year three. (Table 3) Interestingly, cows positive for CWD were significantly more likely to be found pregnant than CWD negative cows (73.4% vs. 59.1%, respectively, $P < 0.05$). (Table 3)

Body condition scores among elk with different PRNP backgrounds and CWD status

In a previous study, cervids with *PRNP* alleles found to be less susceptible to CWD were anecdotally characterized as having 'less than optimal' body condition [32]. Meanwhile, poor body condition is often associated with animals terminally affected with CWD [1]. In an effort to determine whether body condition scores correlated with either *PRNP* genotype or CWD status, we evaluated BCS for 354 animals in year two and 150 animals in year three of the study.

Using three-factor ANOVA analysis, no correlation was observed between BCS and either sex or genotype. Perhaps not surprisingly, a correlation was observed between CWD status and BCS, with CWD positive animals generally scoring lower than those found negative by antemortem testing. (Table 4 and Supplementary Table 2)

Annual survival of young elk with different PRNP backgrounds

The same study referenced above has also suggested, anecdotally, that cervids with less susceptible *PRNP* alleles may be faced with poor young recruitment [32]. We therefore considered the annual return rate of calves and yearling elk in an effort to identify any genotype-specific correlations.

We found that calf return rates were not significantly different across elk *PRNP* genotypes, with 51 of 76 132MM, 42 of 68 132ML, and 12 of 19 132LL calves

Table 3. Pregnancy status among cow elk of various *PRNP* genotypes (3a) and CWD status (3b). Pregnancy was determined by rectal palpation, with *PRNP* genotype confirmed by sequencing and CWD status confirmed by antemortem rectal biopsy testing.

	132MM		132ML		132LL	
Study Period	Pregnant	Open	Pregnant	Open	Pregnant	Open
Year 2	46	21	75	30	5	5
Year 3	13	17	19	14	0	5
Cumulative	59	38	94	44	5	10

	CWD positive		CWD Negative	
Study Period	Pregnant	Open	Pregnant	Open
Year 2	46	17	80	39
Year 3	12	4	20	32
Cumulative	58	21	101	71

Table 4. Correlation of sex, CWD status, and Genotype with Body Condition Scores. Body condition scores were collected from elk by manual palpation during sampling in Study Years 2 and 3, and were correlated to sex, CWD status, and *PRNP* genotype using a three-way ANOVA analysis with regression. A significant correlation was observed between CWD status and body condition score.

Factor(s) Considered	p-value
Sex	0.742046
CWD Status	0.008674
Genotype	0.079612
Sex and CWD Status	0.780121
Sex and Genotype	0.670946
CWD Status and Genotype	0.078185
Sex, CWD Status, and Genotype	0.708582

returning for a second year of sampling. The same held true for yearling elk, where 49 of 75 132MM, 45 of 64 132ML, and 12 of 16 132LL animals returned in the following year for sampling. (Figure 3)

Discussion

While reports on the management of chronic wasting disease in wild deer and elk are many and varied [33–43], rare is the case presented for managing the disease in farmed cervids. Almost without exception, farmed cervids are immediately placed under quarantine and eventually depopulated when CWD is discovered on site [12]. This manuscript reports our efforts to manage CWD in a large elk herd, in a controlled setting with endemic CWD, through the use of annual live animal testing and targeted culling of CWD positive cows. Although the herd owners were presented with additional management directives, including culling of CWD positive bulls and those animals positive by an amplification assay (RT-QuIC), they were not implemented due to concern regarding its potential impact on hunting revenue. Ultimately, we could not completely evaluate our management practices, as the herd was slowly depopulated after the final sampling period due to the financial burden brought by the disease.

Over the three-year study, more than 550 animals were sampled, including calves, bulls, and cows – many on multiple occasions. The study design provided information on several aspects of this disease that had not been explored or reported previously in this species, including the longitudinal evaluation of elk by RT-QuIC on samples collected antemortem. Importantly, we found that the RT-QuIC assay provided a significant improvement in sensitivity over conventional IHC in this study, identifying positive animals irrespective of either follicle count or *PRNP* genotype – two factors known to affect RAMALT IHC sensitivity [25–27]. Our sensitivity estimates for these two assays in elk (52% for IHC and 76% for RT-QuIC) were comparable to those in a previous study in whitetail deer [23]. A more limited study in elk found that RT-QuIC and IHC sensitivities were similar to one another (77%, 21); however, conditions were ideal for immunohistochemical detection in the farmed herd reported in that study – including high biopsy follicle counts, a high frequency of the 132M allele, and many

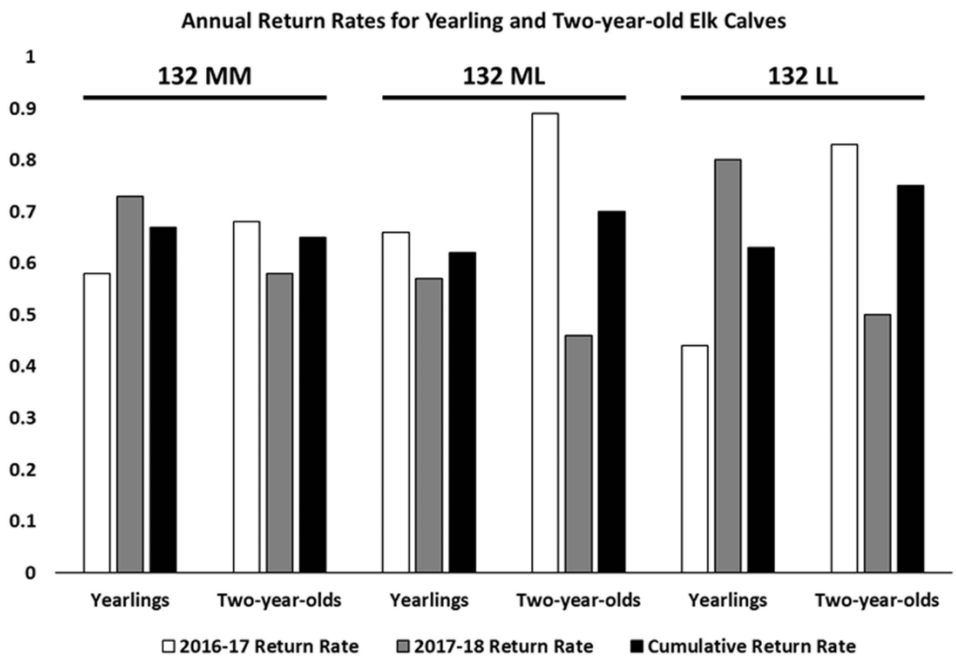

Figure 3. Annual return rates for yearling and two-year-old elk calves. Return rates of young elk were calculated based on animals present at inventory across the 2016–2018 sampling periods. Genotypes were confirmed by sequencing the *PRNP* gene.

of the animals in advanced stages of disease. Ultimately, elk in the present study identified as CWD positive by either the RT-QuIC assay or IHC were 3.6 times more likely to die in the field over the course of a year than their CWD negative cohorts. These dramatically different survival patterns are largely comparable to those reported for whitetail and mule deer in CWD endemic areas [31,44]. The few positive elk that did return were nearly 3.5 times as likely to have the 132ML genotype and were inevitably positive again antemortem, and no animal diagnosed as having CWD was found to have survived a second year.

Our study also allowed us to explore the relationship between *PRNP* genotype and (1) age at first detection of CWD infection, (2) pregnancy rates and (3) year over year calf and yearling survival. In addition to confirming reduced susceptibility in elk with the 132ML and 132LL genotypes, nearly one quarter that of 132MM elk, we report that animals with the 132ML genotype were found to be CWD positive a full year later, on average, than their 132MM counterparts. Pregnancy rates between 132MM and 132ML cows were similar, though elk pregnancy rates can be highly variable and affected by factors outside of the scope of the present study, including population density [45]. The relatively low numbers of 132LL animals made each of these comparisons difficult – especially when considering pregnancy rates and the unfortunate fact that 132LL cows were not allowed to be bred between the second and third years of sampling. Annual calf and yearling survival rates in 132MM, 132ML, and 132LL animals were similar, although we could not assess survival for the first nine months of life due to the timing of sample collections, an admitted limitation in our assessment.

Lastly, we were able to evaluate the relationships between body condition scores (BCS) and both *PRNP* genotype and CWD status. Using a three-factor ANOVA approach, we found no significant correlation between BCS and sex; and although the differences observed in BCS across genotypes were not significant, there was a trend towards higher body condition scores in 132ML animals that may warrant further investigation. Perhaps not surprisingly, a correlation was observed between CWD status and body condition score – with CWD positive animals generally scoring lower. Although the differences were significant, it is likely that BCS may remain an imperfect factor in the clinical differentiation of CWD positive and negative individuals.

Taken together, our findings provide insight into an important question that has been posed regarding CWD and less susceptible *PRNP* alleles: whether these rarer alleles are somehow associated with an evolutionary fitness disadvantage [32]. We found no evidence over the course of the project to suggest that 132ML elk had anything but a higher level of fitness than 132MM animals, especially in the face of a high level of endemic CWD prevalence. Less data was available on 132LL elk, and further investigations are warranted to better assess their level of susceptibility and any potential fitness costs that may be associated with this genotype.

Although we were unable to fully evaluate our management goals for this particular herd, future studies on the small-scale management of CWD in closed herds may be improved by revisiting several important aspects of the present study. First, while the antemortem collection of RAMALT tissue is relatively straightforward for both those collecting samples and those being collected from, more problematic samples like tonsil biopsies may provide a higher level of sensitivity, allowing for a more effective removal of clinical and preclinically infected animals [46,47]. Secondly, stressing the complete and timely removal of all animals identified as CWD positive will be important in limiting horizontal transmission. Third, it may prove useful to more effectively implement a genetic strategy as it relates to the *PRNP* gene – through the removal of more susceptible genotypes in favour of the propagation of those found to be less susceptible. Past studies [39,48], and studies ongoing, may provide additional insight into the utility of the genetic-based management of CWD. Lastly, it will be important to adequately address prions shed into the environment. While this aspect is inherently the most difficult to address, there are several potential approaches that may show some benefit, including pasture rotation and reduction of animal density, practices which have been used to effectively reduce the prevalence of other important diseases, including prion diseases [49–52].

Despite the failures in our approach, the present study provides firm data on the relative sensitivity of the RT-QuIC assay compared to conventional immunohistochemistry of RAMALT tissues, and these findings are very likely to extend to tissues collected post-mortem. The study also presents important data points on CWD susceptibility, survival, and disease progression in animals with different *PRNP* genotypes. It is our hope that this study will serve as a resource for game biologists seeking to model the effects of CWD on elk herds. The mistakes and hurdles encountered will almost certainly allow for the improvement of CWD management strategies in farmed elk, should the opportunity arise again in the future.

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Disclosure of Potential Conflicts of Interest

No potential conflict of interest was reported by the authors.

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