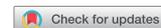


RESEARCH PAPER



Chronic wasting disease management in ranched elk using rectal biopsy testing

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ABSTRACT

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) affecting members of the cervid species, and is one of the few TSEs with an expanding geographic range. Diagnostic limitations, efficient transmission, and the movement of infected animals are important contributing factors in the ongoing spread of disease. Managing CWD in affected populations has proven difficult, relying on population reduction in the case of wild deer and elk, or quarantine and depopulation in farmed cervids. In the present study, we evaluated the effectiveness of managing endemic CWD in a closed elk herd using antemortem sampling combined with both conventional and experimental diagnostic testing, and selective, targeted culling of infected animals. We hypothesized that the real-time quaking-induced conversion (RT-QuIC) assay, a developing amplification assay, would offer greater detection capabilities over immunohistochemistry (IHC) in the identification of infected animals using recto-anal mucosa associated lymphoid tissue (RAMALT). We further sought to develop a better understanding of CWD epidemiology in elk with various *PRNP* alleles, and predicted that CWD prevalence would decrease with targeted culling. We found that RT-QuIC identified significantly more CWD-positive animals than IHC using RAMALT tissues (121 vs. 86, respectively, out of 553 unique animals), and that longstanding disease presence was associated with an increasing frequency of less susceptible *PRNP* alleles. Prevalence of CWD increased significantly over the first two years of the study, implying that refinements in our management strategy are necessary to reduce the prevalence of CWD in this herd.

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Introduction

Chronic wasting disease, a transmissible spongiform encephalopathy (TSE) found in farmed and wild populations of several cervid species, was first identified half a century ago in captive mule deer (*Odocoileus hemionus*) in a research facility in the Colorado foothills [1]. Since then, the disease has been reported in five different cervid species in 23 additional states, two Canadian provinces, the Republic of Korea, and Norway, with many of these novel foci arising since the turn of the 21st century [2–4]. Like other members of the TSE family, chronic wasting disease (CWD) is the result of an infection with a misfolded variant of the normal cellular prion protein (PrP^{res} and PrP^C, respectively), which produces a slowly progressive neurologic disease culminating in death. Unlike other TSEs, local and regional prevalence of CWD in endemic areas is slowly increasing with an ever-expanding geographic range [2] [5–9], a result of the difficulties presented with managing a

disease having a long incubation period and both efficient horizontal and environmental transmission pathways – through animal to animal contact or exposure to infected carcasses [10], excreta [11–13], or potentially vegetation present on the landscape [14].

Currently available testing approaches for diagnosing CWD in cervids involve the postmortem analyses of the medial retropharyngeal lymph nodes (RLNs) and the obex region of the brainstem using immunohistochemistry (IHC) or enzyme immunoassay (EIA) [15]. These conventional assays, considered the “gold standard” for determining CWD infection status, primarily take advantage of the partial resistance of PrP^{res} to harsh physical treatments. Treatments such as proteinase K digest PrP^C, leaving the abnormally folded protein behind where it is recognized through antibody-antigen complexing. Although both assays are considered highly sensitive and specific in deer

and elk, several studies have found that test sensitivity may be imperfect [16–18]. This is partly a result of the very low levels of PrP^{res} present in early stages of infection, although the harsh pre-treatments may additionally obliterate some of the more sensitive isoforms of infectious PrP^{res} [19,20]. These findings have in turn spurred the development of amplification assays like the serial protein misfolding cyclic amplification assay (sPMCA) [21] and real-time quaking-induced conversion (RT-QuIC) [22,23], which are thought to amplify low levels of PrP^{res} *in vitro*, through templated conversion, to levels which may be readily observed on their respective platforms. Conventional and experimental diagnostic approaches have increasingly focused on antemortem testing, using peripherally accessible tissues like recto-anal mucosa associated lymphatic tissue (RAMALT) [18,24,25]. There is hope that antemortem tests with adequate sensitivity could play a critical role as surveillance tools for screening animals under quarantine, and more importantly if employed prior to the movement of animals or their byproducts, to prevent the further spread of CWD.

Improvements in the identification of infected animals over the past two decades have helped demonstrate that not all deer and elk are equally susceptible to CWD [26–28]. Susceptibility in deer and elk has been linked to specific alleles of the normal cellular prion protein, encoded by the *PRNP* gene. Less susceptible cervid alleles include the 96S allele in white-tailed deer (*Odocoileus virginianus*), the 225F allele in mule deer, and the 132L allele in North American elk (*Cervus elaphus elaphus*). Commonly, the frequencies of these less susceptible alleles, including the 132L allele in elk, are rare in wild populations – in most studies making up less than 18% of the population [25,28,29]. The reasons for their low frequencies in cervid populations are unknown, although it is assumed that these animals may face some fitness disadvantage which keeps the allele at its present levels. What is known is that elk with the less susceptible 132L allele, for example, are significantly less likely to show evidence of infection in the obex or RLN than their wild type 132M counterparts; moreover, those that are infected are often in much earlier stages of the disease and may have unique PrP^{res} banding patterns on Western blot [25,27,29]. CWD-infected animals considered less susceptible, including 132ML or LL elk, were also less likely to be positive for CWD in peripherally-accessible tissues like RAMALT and nasal brush collections [18,25]. These findings helped to better characterize this reduced susceptibility as a delayed progression of disease. While amplification assays may prove more sensitive in RAMALT and other tissues, delayed disease progression remains an important limiting factor in the reliable antemortem identification of infected animals.

Diagnostic limitations, efficient horizontal and environmental transmission, and the movement of infected animals, their carcasses and potentially even cervid byproducts, are three components of a triumvirate which make management of CWD a considerably difficult proposition [5,8,30,31]. Several different strategies used in the management of other infectious diseases of veterinary importance have been employed in North America in efforts to manage CWD, including local population reduction in the case of free-ranging deer and elk [9,32], and the quarantine and depopulation of infected farmed cervid herds [18,24,25,33]. These strategies are meant to help eliminate or reduce horizontal and environmental transmission and prevent the movement of exposed or infected animals, or at the very least slow their dispersion. Previous management attempts have met with mixed success and failure, and despite intense efforts, CWD continues to escape its confines in its insidious march across North America and beyond. Antemortem test and cull strategies have only been attempted on a limited basis in free-ranging populations, despite optimism that early detection and removal of infected animals may help in managing the disease [31,34]. The utility of this approach in managing CWD in farmed cervids has not, to this point, been evaluated.

In the present study, we sought to determine the feasibility of managing CWD in ranched North American elk through antemortem testing and targeted culling of infected animals over a two-year period. The goals of the study were severalfold: first, to compare the longitudinal detection capabilities of conventional IHC and RT-QuIC for antemortem diagnosis of CWD using RAMALT biopsies; second, to better understand the epidemiology of CWD in elk with different *PRNP* alleles; and finally, to examine the impact of a limited test and cull strategy on CWD prevalence in a controlled population. We had three corresponding hypotheses associated with these goals: 1) RT-QuIC would prove more capable than IHC in the antemortem detection of infected animals; 2) the selective pressure placed by CWD on the 132M allele would drive an increased frequency of the less susceptible 132L allele; and 3) identifying infected animals antemortem and removing them would help to lower CWD prevalence in the herd over the course of the study. Conclusions for each of these hypotheses would provide valuable insight into the epidemiology of this disease and importantly help refine future management strategies.

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 [35].

Study area and population

This study was conducted in a 3500-acre (14 km²) fenced-in area of private land in Colorado. Elevation ranged from 2,000 m to 2,400 m with mixed habitat consisting of grazing land, dense pine forest, several natural streams, and man-made reservoirs – not unlike other areas of Colorado with endemic CWD [34,36]. Chronic wasting disease was initially identified on the premises in 2004, and prevalence has slowly increased since then, ranging from 16–26% using harvest data collected from 115 animals in 2014 and 2015 (Table 1). Prevalence outside the fence is unknown, though presumed to be greater than 5–10% in elk and mule deer [37,38]. The managed herd consisted of 4–500 adult and yearling animals. Cow elk range in age from 1–16 years, and bulls range from 1–8 years of age. Animals were handled once yearly in the late winter, when they were captured as a group in a large grazing pasture and run through a modern handling system for inventory, sample collection, and routine medical treatments. At that time, animals were identified using detailed information from ear tags (including RFID chips) and tattoos. Unmarked animals were tagged and tattooed for future identification.

Study design

During the winter inventory of 2016, a range of samples were collected for genetic analysis and antemortem CWD testing from 387 adult elk, including blood and rectal biopsies as described below. In calves less than one year of age (n=78), blood samples alone were collected for genetic analysis. After sampling, adult cows were retained in the original capture pen until CWD testing was complete (approximately

5–7 days). All bulls were turned out into a 500-acre winter pasture regardless of CWD status, and all calves were released into a 500-acre spring pasture. Once testing was finished, adult cows were again run through the handling system. Cows which were CWD positive by RAMALT IHC were diverted to a separate enclosure for additional sampling, euthanasia, necropsy and postmortem sampling. Cows negative by IHC, including those which were RT-QuIC positive or RT-QuIC suspects, were released into the 500-acre spring pasture with the calves. In the late spring, the bulls were again intermixed with the cows and calves and all were allowed full access to the remainder of the ranch. In the winter inventory of 2017, the remaining animals as well as new additions, totaling 315 adults and 85 calves, were sampled and handled as before.

Antemortem and postmortem sampling

During sample collection, animals were restrained using a conventional large animal squeeze chute. With adequate restraint, blood was collected from the jugular vein and placed into an ethylenediaminetetraacetic acid tetrasodium salt (EDTA) tube. Rectal biopsies were collected using sterile, single use instruments by removing a 2 cm × 2 cm piece of superficial mucosal tissue from the wall of the rectum, approximately 2 cm ad-oral from the mucocutaneous junction of the anus as described previously [25,36]. A 0.5 cm × 0.5 cm subsection of this biopsy was placed into a 1.5 ml microcentrifuge tube and frozen for RT-QuIC analysis, while the remainder was placed into a histology cassette and preserved in 10% neutral buffered formalin for IHC analysis.

Postmortem samples were collected at necropsy from animals euthanized during inventory, and when available from animals which died in the field (e.g. those hunted or dying of natural causes). At a minimum, the obex region of the brainstem and the medial retropharyngeal lymph nodes were collected where available and stored in 10%

Table 1. Historical postmortem testing for CWD in harvested animals on the ranch, including estimated prevalence and 95% confidence intervals.

Year	Number positive postmortem (IHC)	Number tested	Prevalence (95%CI)
2004	2	10	20% (2.5–56%)
2005	1	10	10% (0.3–45%)
2006	2	19	10% (1.3–33%)
2007	0	57	0% (0–6.3%)*
2008	0	39	0% (0–9%)*
2009	1	39	2.6% (0.1–14%)
2010	0	27	0% (0–13%)*
2011	7	33	21% (9–39%)
2012	6	52	12% (4.4–23%)
2013	4	67	6% (1.7–15%)
2014	19	72	26% (17–38%)
2015	7	43	16% (6.8–31%)

*In cases where no positive animals were found, a one-sided 97.5% confidence interval (CI) is presented

neutral buffered formalin for postmortem confirmation of CWD status.

Diagnostic procedures

Immunohistochemistry (IHC)

Rectal biopsies and postmortem samples were evaluated for PrP^{res} immunostaining as described previously [18,24,25], blindly and without information on the index test (RT-QuIC) results. Briefly, immunohistochemical staining for PrP^{CWD} was performed using the primary antibody Anti-prion 99 (Ventana Medical Systems, Tucson, AZ) and then counter-stained with hematoxylin. Positive and negative controls were included in each analysis. Biopsies were considered positive if at least one follicle exhibited PrP^{CWD}-specific staining [39]. In cases where biopsies had fewer than 6 follicles, samples were classified as “insufficient follicles,” unless CWD-specific immunohistochemical staining was observed which would allow them to be classified as CWD positive.

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

Rectal biopsy subsections were prepared as 10% homogenates in phosphate-buffered saline (PBS) and analyzed for PrP^{res} conversion activity. RT-QuIC assays were performed using a truncated form of the recombinant Syrian hamster PrP (SHrPrP, residues 90–231) in pET41b and expressed and purified as previously described [18,25,40]. Rectal biopsy homogenates were first diluted 1:100 in RT-QuIC dilution buffer (0.05% sodium dodecyl sulfate in PBS). Five μ l of this 10^{-2} dilution were then added to 95 μ l of RT-QuIC reaction buffer, consisting of 50 mM NaPO₄, 350 mM NaCl, 1.0 mM EDTA, 10 μ M thioflavin T (ThT), and 0.1 mg/ml truncated Syrian hamster rPrP^C, to yield a final volume of 100 μ l. Each test sample was analyzed blindly, without information on the reference test (IHC) results, and was repeated in triplicate on a single plate. Positive controls, consisting of 5 μ l of a 10^{-3} dilution of pooled CWD positive brain from six experimentally infected white-tailed deer (cervid brain pool 6, CBP6) spiked into 95 μ l of RT-QuIC reaction buffer, were included in triplicate in each experiment. This control has well-characterized rates of amplification in RT-QuIC and has been titrated in transgenic Tg[CerPrP] mice [18,25,41,42]. Negative controls, also repeated in triplicate, consisted of three RAMALT biopsies from known CWD negative deer. Reactions were prepared in black 96-well, optical-bottom plates, which were then sealed and

incubated in a BMG Labtech PolarstarTM fluorimeter at 42°C for 24 hours (ninety-six cycles, 15 minutes each) with intermittent shaking; specifically, 1 minute shakes (700 rpm, double orbital pattern) interrupted by 1 minute rest periods. ThT fluorescence measurements (450 nm excitation and 480 nm emission) were taken every 15 minutes with the gain set at 1800. The relative fluorescence units (RFU) for each triplicate sample were progressively monitored against time with orbital averaging and 20 flashes/well at the 4 mm setting.

Criteria for identification of positive samples was determined *a priori* [18,25]. Briefly, a replicate well was considered positive when the relative fluorescence crossed a pre-defined positive threshold, calculated as ten standard deviations above the mean fluorescence of all sample wells from cycles 2–8. Positive samples were those which crossed the threshold in $\geq 2/3$ replicates; animals with just 1/3 replicates positive were considered “suspects.”

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 as previously described [18,25,26]. PCR products were bidirectionally sequenced (Genewiz, USA), and 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 132 MM, ML, or LL genotypes. Additional, non-coding polymorphisms including C→T at *PRNP* position 72, and A→G at *PRNP* position 321 [29] were catalogued but not considered in the present analysis.

Data analysis

Statistical analysis of various components of the data set were analyzed using GraphPad Prism 7.0 software. A two-tailed Fisher’s exact test was used to compare the number of positive animals identified by IHC and RT-QuIC, the *PRNP* frequency variation from historical studies and the prevalence of CWD among different *PRNP* genotypes in the present study, the return rates of infected and uninfected animals, and the CWD positive conversion rates seen in animals classified as RT-QuIC suspects. A two-tailed Mann-Whitney test was used to compare the age structures of total and CWD affected populations with different *PRNP* alleles. *P* values below 0.05 in all cases were considered significant.

Results

Antemortem identification of CWD infected animals using RAMALT biopsies is improved with RT-QuIC

Antemortem detection of CWD infection using RAMALT IHC

During the 2016 sampling period, RAMALT biopsies from 387 adult elk over 2 years of age were collected and analyzed by IHC. Immunohistochemistry identified 33 positive animals – 20 cows and 13 bulls, for an apparent prevalence of 8.5% (Tables 2 and 3). Subjectively, these animals rarely showed symptoms of infection, with just a single cow presenting with cachexia and severe obtundation. One of these IHC positive animals, a bull, was considered an RT-QuIC suspect, while a single IHC positive cow was negative by RT-QuIC. Immunohistochemistry results were returned in an average of 5.7 days. Nineteen positive cows (one IHC positive only, 17 IHC and RT-QuIC positive, and one RT-QuIC positive only) were euthanized, with obex and RLN tissues collected postmortem for confirmation of CWD infection. All were positive by IHC in both tissues. Two IHC positive cows were inadvertently released into the winter feeding pasture with the bulls. Neither of these cows nor any of the IHC positive bulls – a total of 15 IHC positive animals – returned for the 2017 sampling period. None of these animals were hunted and they were presumed to have died in the field, however postmortem tissues could not be collected for confirmation of infection.

During the 2017 sampling period, RAMALT biopsies from 315 adult elk over 2 years of age were collected and analyzed by IHC. Two hundred sixty-five of these were previously sampled in 2016, while 50 animals were not sampled in 2016 due to their age (n = 47) or absence from inventory (n = 3). Of the 315 animals sampled, fifty-three were considered IHC-positive – 34 cows and 19 bulls, for an apparent prevalence of 17%. Of these, two were RT-QuIC negative and two were considered RT-QuIC suspects. Results were returned in an average of 5 days. Thirty-two of these animals (thirty-one IHC positive cows and one IHC-negative, RT-QuIC positive bull) were euthanized; all were found to be IHC positive postmortem in obex, RLN, and/or tonsil. The 18 remaining IHC positive bulls, three IHC positive cows, and all IHC negative, RT-QuIC positive animals were released.

In both years, biopsies were collected over three to four days, with sampling times of 3–4 minutes per animal. Biopsies were sent in separate daily batches for testing by IHC, with an average turnaround time across both years of 5.4 days.

Antemortem RT-QuIC identification of CWD positive animals in RAMALT

Subsections of biopsies collected in 2016 and 2017 sampling periods were also analyzed blindly by RT-QuIC for evidence of prion seeding activity. Among the 387 samples collected in 2016, 63 animals were identified as positive by RT-QuIC, including 42 cows and 21 bulls, for an

Table 2. Summary of antemortem and postmortem testing by antemortem test result and sex. 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. Postmortem samples were collected either following euthanasia or when available from animals harvested in the field, to confirm CWD status of animals identified antemortem. Animals from the first year of sapling which did not return for the second year of the study were presumed to have died in the field, with no postmortem data available for testing. Postmortem test results are not yet available from the year two study period. NA: postmortem results were not available from bull elk which were released and did not return for sampling in the second year of the study.

Antemortem Test Result	Study Year One (2016)					Study Year Two (2017)				
	Sex	Number	Postmortem Test Result		Returned	Antemortem Test Result				
			CWD (+)	CWD (–)		IHC(+) only	IHC(+), RT-QuIC (+)	RT-QuIC(+) only	IHC(–), RT-QuIC Suspect	IHC(–), RT-QuIC (–)
IHC(+) only	Bull	1	NA	NA	0					
	Cow	1	1	0	0					
IHC(+), RT-QuIC(+)	Bull	12	NA	NA	0					
	Cow	19	17	0	0					
RT-QuIC(+) only	Bull	9	NA	NA	2	0	1	1	0	0
	Cow	23	1	0	8	1	7	0	0	0
IHC(–), RT-QuIC Suspect	Bull	10	0	2	8	0	3	0	1	4
	Cow	7	1	0	6	0	2	1	0	3
IHC(–), RT-QuIC(–)	Bull	115	2	22	78	0	13	5	3	57
	Cow	190	2	9	163	2	20	8	5	129
Untested Calves	Bull	42	0	7	26	0	2	2	2	20
	Cow	36	0	6	21	1	1	1	0	19
Total	Bull	189	2	31	114	0	19	8	6	81
	Cow	276	22	15	198 (+3) [*]	4	30	10	5	152

^{*}Three adult cows which were not sampled in study year one presented for testing in year two.

Table 3. Yearly summary of antemortem and postmortem 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 infection. Postmortem samples were collected either following euthanasia or when available from animals harvested in the field, to confirm CWD status of animals identified antemortem. Animals from the first year of sampling which did not return for the second year of the study were presumed to have died in the field, with no postmortem data available for testing.

Study Period	132 <i>PRNP</i>	Number Tested		Untested calves	Antemortem test results				Postmortem IHC results				
		Bulls	Cows		IHC(+)	IHC(+), RT-QuIC(+)	RT-QuIC(+)	IHC(-), RT-QuIC Suspect	IHC(-), RT-QuIC(-)	CWD (+)	CWD (-)	NT	Returned
Year 1	MM	51	101	31	0	22	17	6	107	14	10	48	111
	ML	80	129	38	1	10	15	9	174	10	26	35	176
	LL	16	10	9	0	0	0	2	24	0	5	5	25
Year 2	MM	38	74	45	2	36	5	3	66				
	ML	63	115	30	0	14	11	8	145				
	LL	13	12	10	0	1	0	0	24				

Postmortem results and returning numbers for year two are incomplete until findings from the 2018 sampling period are available.

apparent prevalence of 16% (Tables 2 and 3). Of these animals, 31 were also IHC positive, while 7 had an insufficient number of follicles for interpretation. Thirty-two of these RT-QuIC positive animals had been considered IHC negative, or otherwise “not detected.” There were 18 animals classified as RT-QuIC suspects, including one which was considered positive by IHC. Results from RT-QuIC analysis were returned in an average of 3.8 days. Of the 63 RT-QuIC positive animals and 18 RT-QuIC suspect animals, 19 were those cows discussed above which were euthanized, the remainder, including IHC positive bulls and IHC negative, RT-QuIC positive animals, were released. Thirty-six out of 46 RT-QuIC positive animals (21 of 31 which were IHC negative) released after sampling failed to return for the 2017 sampling period, none of these animals were hunted and they were presumed to have died in the field. No postmortem data could be collected from these animals to confirm infection.

Among the 315 animals tested in 2017, there were 67 RT-QuIC positive animals, including 40 cows and 27 bulls, for an apparent prevalence of 21%. Results from RT-QuIC analysis were returned in an average of 3.8 days. Of the 67 RT-QuIC positive animals, 49 were also IHC positive, while six had insufficient follicles for classification. The remaining 12 were considered IHC negative. Nine of the 67 were those elk that were RT-QuIC positive in 2016 which had returned in 2017; all were subsequently RAMALT positive by IHC on repeat testing. Each of these animals were euthanized and evaluated postmortem, and all were positive in the obex and RLN. A single cow which was RT-QuIC positive in 2016 was subsequently IHC positive only in 2017; she was inadvertently released into the winter pasture after sampling. RT-QuIC positive animals made up 27 of 31 of the IHC positive cows which were euthanized, all of which were confirmed CWD positive postmortem. A bull which was RT-QuIC positive and had insufficient follicles by IHC was also euthanized and found to be CWD positive postmortem. Twenty-one IHC and RT-QuIC

positive animals were released back onto the ranch (19 bulls and 2 cows), as were as 17 IHC negative, RT-QuIC positive animals (7 bulls and 10 cows).

As with IHC, during each study period, biopsies were collected and sent in daily batches. The average turnaround time for RT-QuIC testing was 3.8 days across the 2016–2017 study periods. Over the course of both study years, twenty eight experimental plates were analyzed. Results from five plates were disqualified and the experiments repeated; three plates prepared simultaneously had positive controls which failed to amplify, one plate had a single false positive replicate well from one negative control demonstrating amplification, and a third suffered from a technical error with the plate reader mid-experiment.

The fate of RT-QuIC suspect animals

In the 2016 sampling period, there were 18 animals considered RT-QuIC suspects. One was positive by IHC on RAMALT – a bull – and was presumed to have died in the field after failing to return for the 2017 sampling period (Tables 2 and 3). Two were hunted in the fall of 2016 and were found to be IHC negative postmortem. One animal was reported as showing clinical signs suggestive of CWD immediately prior to the 2017 sampling period and was euthanized – testing positive postmortem by IHC in the brain, lymph nodes, and tonsil. The remaining 14 animals all returned for the 2017 sampling period. Five of these were found to be RT-QuIC and IHC positive, a sixth was RT-QuIC positive with insufficient follicles reported on IHC, and a seventh was once again an RT-QuIC suspect, though IHC negative. Seven of fourteen were RT-QuIC and IHC negative.

CWD prevalence in previously IHC negative, RT-QuIC negative elk

A total of 305 animals were not considered CWD positive or suspects by either testing approach in the 2016 sampling period (Tables 2 and 3). Ten were found dead

in the field soon after sampling and tested negative by postmortem IHC. Twenty-five were hunted, and of these, four were found to be positive by postmortem IHC. Twenty-nine of the remaining 270 did not return for the 2017 sampling period, and were presumed to have died in the field. Of the 241 which did return, one was IHC positive only, a second was considered IHC positive and an RT-QuIC suspect. Thirty-three of the returning animals were positive by both IHC and RT-QuIC. Thirteen animals were RT-QuIC positive only, with three having insufficient follicles on IHC, and another eight were considered RT-QuIC suspects. The apparent CWD prevalence in 2017, among previously negative animals, was 15% and 19% by IHC and RT-QuIC, respectively. There were a total of 186 animals which were negative in both years of the study.

One year incidence of CWD among negative or previously unsampled animals

Fifty animals were sampled in 2017 which had not been tested in 2016, including 47 two-year old animals initially identified during the 2016 inventory and 3 previously unsampled adults. One of these 50 animals was IHC positive and an RT-QuIC suspect by RAMALT testing, while three were positive by both IHC and RT-QuIC. Four animals were RT-QuIC positive only, with one having insufficient follicles for IHC analysis, and there were an additional two considered RT-QuIC suspects. Forty of these animals were negative by both assays. As noted above, there were 35 previously negative animals for which postmortem data was available between the 2016 and 2017 sampling period, and of these 4 were CWD positive. There were 241 animals which were negative by both assays in 2016 which had returned during the 2017 sampling period. Forty-seven were positive by one or both assays, eight were RT-QuIC suspects, and 186 were again negative by IHC and RT-QuIC. When considering previously untested calves and negative adults, the cumulative yearly incidence of CWD between the 2016 and 2017 sampling periods was 18% based on IHC or RT-QuIC (59 positive among 326 animals with ante- or post-mortem test results available).

One year mortality rates of CWD positive elk identified through antemortem testing

Of the 15 IHC and RT-QuIC positive animals that were released following the 2016 sampling period, none returned in the second year of the study, for a one year mortality rate of 100%. Twelve of these animals were 132 MM and three were 132 ML. Out of 31 IHC negative, RT-QuIC positive animals released to the field, only 10 returned for the second year's inventory, giving a mortality rate of 68% – 4.8 times higher than the baseline

mortality rate of 14% among CWD negative, non-hunted animals (10 animals found dead in the field and 29 which did not return for sampling out of 280 non-hunted animals). Seventeen of 31 were 132 MM, and the remainder were 132 ML. Of those that returned, four were 132 MM and six were 132 ML. Genetic analyses are discussed in further detail below.

In summary, 553 unique animals were sampled across both years of the study, with 437 being tested for CWD antemortem. One hundred thirty-one were CWD positive by IHC or RT-QuIC, either antemortem or postmortem (30%), and only rarely were these positive animals showing clinical signs of disease. Antemortem IHC identified 86 RAMALT positive animals across both sampling periods, while RT-QuIC identified significantly more, with 121 unique positive animals in 2016 and 2017 ($P < 0.0001$, two-tailed Fisher's exact test). Considering IHC and RT-QuIC positive animals only, across both years of the study the positive percent agreement (PPA) between the two tests was 94% (95% CI: 87–98%) while the negative percent agreement (NPA) was 89% (95% CI: 86–91%). Thirteen of 49 animals positive by RT-QuIC alone were found to have insufficient follicles for IHC diagnosis, suggesting that follicles may not be necessary for PrP^{tes} amplification in RT-QuIC. Most notably, of the 46 animals identified as positive by either IHC or RT-QuIC in 2016 which were released, only ten returned for the 2017 study period. Each of these ten animals were RAMALT positive by RT-QuIC only in 2016, and each were subsequently positive in 2017 by antemortem IHC. The return rate for non-hunted, IHC or RT-QuIC positive animals (10/46, or 21.7%) was significantly lower than that of non-hunted, CWD negative animals (241/270, 89%, $P < 0.0001$, two-tailed Fisher's exact test). The rate at which non-hunted animals converted from RT-QuIC suspects to IHC or RT-QuIC positive between years was 39% (7/18), significantly higher than that of previously negative animals (47/241 or 19.5%, $P = 0.0238$, two-tailed Fisher's exact test), implying that the “suspect” classification may be a fair predictor of CWD status.

A high frequency of the 132L allele may be found in herds with long-standing CWD endemicity, as CWD is found at a higher frequency in animals with the 132M allele

Frequency of the 132M and 132L alleles

In the 2016 sampling period, a total of 465 animals, including 387 adults and 78 calves, had blood collected for PRNP analysis. Among these 465 animals sampled in the first year, 183 were homozygous for the 132M allele (39%), 247 were 132 M/L heterozygous (53%), and the remaining 35 were 132L homozygous (7.5%). The

the 42 CWD positive 132 MM animals in year one, the average age was 4.7 years. There were 209 132M/L heterozygous animals with an average age of 6.3 yrs. Of the 27 CWD positive 132M/L heterozygous animals, the average age was 5.7 yrs. There were 26 132LL homozygous animals, all CWD negative, with an average age of 4.9 yrs (Table 4a).

In the 2017 study period, there were 112 animals homozygous for the 132M allele, with an average age of 5.3 yrs. Of the 43 CWD positive animals, the average age was also 5.3 yrs. Among the 208 uninfected 132M/L heterozygous animals, the average age was 5.8 yrs. The average age of CWD positive 132M/L homozygous animals was 5.7 yrs. There were 35 132LL homozygous animals with an average age of 3.7 years; the single CWD positive 132LL homozygous animal was 11 years of age (Table 4b).

Age structures between genotypes, based on total and affected populations, were compared using a two-tailed Mann-Whitney test. In the first year, there was no significant difference between the age structures of 132 MM and 132ML populations ($P = 0.59$). Differences in age structures became more apparent in year 2, although differences between the 132 MM and 132 M/L populations were still not significant ($P = 0.36$). When comparing the age structures of CWD affected animals, based upon age of first diagnosis across both study years, the findings were again not significant ($P = 0.39$). The age structures of both 132 MM and 132ML subpopulations were however significantly different than the 132LL subpopulation ($P < 0.05$ in both study years compared to both 132 MM and 132ML populations).

One year survivorship of uninfected and infected animals with various PRNP alleles

Across the two study years, 132 MM homozygous animals which tested CWD negative had a 79% return rate (89/113), including those animals which were hunted. Homozygous 132 MM animals which tested CWD positive by antemortem IHC or RT-QuIC had a return rate of 10% (4/39). Heterozygous 132 M/L animals which tested CWD negative, included those animals which were hunted, also had a 79% return rate (145/183), although the rate of return for those that tested positive was 23% (6/26). The one year return rate for 132LL homozygous animals, including those that were hunted was 71% (25/35). The second-year return rates among the various alleles were not significantly different, either considering animals which were uninfected ($P = 0.58$, chi-squared test) or animals which were infected ($P = 0.1809$, two-tailed Fisher's exact test), although as the study continues, differences in return rates may become more significant among these groups.

In summary, the frequency of the 132L allele was higher than expected in this herd, representing over 33.4% of the all animals sampled, including calves. The cumulative frequency of this allele in three previous reports was 14.9% [25,28,29], significantly lower than that of that of the present study ($P < 0.0001$, two-tailed Fisher's exact test). Animals homozygous for the 132M allele faced the highest relative risk of being diagnosed as CWD positive, a risk 2.4 times that of 132 M/L homozygous animals and nearly 16 times that of 132LL homozygous animals. Chronic wasting disease-affected age structures and return rate variances were not significantly different between elk genotypes, however differences may become more apparent among these groups with additional sampling years.

A limited test and cull strategy yields no immediate reduction in CWD prevalence

CWD prevalence in the 2016 and 2017 study periods based on antemortem RAMALT sampling

In the 2016 sampling period, the apparent prevalence of CWD among the 387 sampled animals, based on combined IHC and RT-QuIC findings, was 16.8% (65/387) (Tables 3 and 4a). Previous studies have shown that RAMALT testing in elk offers a sensitivity of approximately 80%, meaning the actual prevalence was likely higher. The prevalence in bulls was 15%, while the prevalence among adult cows was 17.9%. There were 78 calves who were untested for CWD in 2016, 47 of these animals survived to be tested during the 2017 sampling period.

In the 2017 sampling period, 315 adult animals were biopsied and tested (Tables 3 and 4b). Ten of these animals (eight cows and two bulls) were previously identified as CWD positive in 2016. The apparent prevalence during the second year of the study was 22.5%. Among bulls, the prevalence was 23.7%, while prevalence in cows was 21.9%. There were 85 calves which were not tested for CWD.

Over the two-year study period, there were 437 unique animals tested for CWD antemortem. The apparent prevalence among those tested, across both years of the study, was 29.5%. Overall prevalence in bulls was 26.9% (47/175), which was not significantly different from the prevalence among cows 29.8% (78/262, $P = 0.52$, two-tailed Fisher's exact test).

Population decline across study periods and causes of death where available

Between the first and second year of the study, the herd lost 153 of 465 animals, including 31 calves and 122 adults (Tables 2 and 3). Eighty-five new calves were sampled in the 2017 study period and there were 3 newly

sampled young adults, resulting in a net loss of 65 animals. Of the 153 animals lost between 2016 and 2017, there were 19 animals euthanized after testing positive for CWD (12.4%). Thirty-six animals diagnosed with CWD were lost to follow-up and were presumed to have succumbed to disease (23.5%). Forty-five negative animals, including calves too young to test, were lost to follow-up (29.4%). Twenty-six animals, including twelve calves, died very soon after the sampling period (17%). Necropsies on many of these animals revealed they were in poor body condition, with depleted fat reserves in the peritoneal cavity and bone marrow. All were either CWD negative postmortem ($n = 14$), or less than one year of age and not tested. A total of 28 animals were hunted during the fall hunting season (18.3%), with four of these animals testing positive for CWD.

In summary, after one year of a management strategy utilizing antemortem RAMALT biopsy and selective culling, the prevalence of CWD on site increased by nearly 6%. Prevalence was somewhat higher in cows over the course of the study compared to bulls, a finding which was not significant. The population declined sharply from 465 animals to 400 animals over the first two years of the study, with CWD-associated deaths making up 35.9% of the 153 animals lost between 2016 and 2017 sampling periods. Because the sensitivity of RAMALT IHC is 80% or less, it seems likely that some fraction of the IHC and RT-QuIC negative animals which were lost to follow-up were also subclinically infected at the time of sampling in 2016, as indicated by the four of 28 hunted animals which tested positive. It seems plausible that some of these incorrectly classified animals could have succumbed to disease prior to the 2017 sampling period.

Discussion

Of the known transmissible spongiform encephalopathies, including scrapie of sheep, bovine spongiform encephalopathy, and human Creutzfeldt-Jakob disease, chronic wasting disease is the only TSE which is currently expanding in both geographical range and prevalence [43–47]. Management strategies to this point have varied when considering farmed and free-ranging cervids. In farmed deer and elk, quarantine and whole herd depopulation has been the standard management approach. While this strategy has largely been successful, there remains an unknown level of environmental contamination which effectively renders the properties unusable for cervid ranching for the foreseeable future [10,48,49]. In free-ranging deer and elk, several attempts at herd reduction have been undertaken, which may locally dampen the horizontal transmission and

environmental accumulation of CWD in the short term, though almost inevitably disease prevalence has been found to rebound, with rare exception [9,30,50,51]. Test and cull strategies have only rarely been attempted in wild cervids, though it has been suggested that this approach may help reduce CWD prevalence [31,34,36]. Prior to the current study, a test and cull management strategy had not been attempted in farmed cervids.

The opportunity to implement this approach in farmed cervids allowed us to pursue unique research avenues into chronic wasting disease epidemiology and diagnostic testing, while at the same time presenting a range of distinct challenges. This study was importantly the first longitudinal study involving a wholly contained group of cervids, which allowed for serial sampling and long-term follow-up in a large number of animals and provided important data on assay accuracy and survival rates. The great majority of the herd has been included in the study, although there are sure to be some number of more feral members who have not yet been sampled. The ability to efficiently sample such a large number of animals to minimize handling and reduce animal stress, combined with the implementation of rapid testing and assay turnaround, was unprecedented among CWD field studies.

The legitimacy of these results – specifically those centered on the detection capabilities of RT-QuIC – may be argued. In the first year of the study, RT-QuIC identified 32 positive animals that were negative by IHC. Many of the IHC and RT-QuIC positive animals, the majority of which were in preclinical stages of infection, were lost to follow-up between years one and two. While postmortem results were not available for most of them, their return rate was 5.6 times lower than that of CWD negative animals. The ten RT-QuIC positive, IHC negative animals which did return in year two were all subsequently positive by IHC. This makes a strong case for the detection advancements offered by RT-QuIC over IHC in antemortem RAMALT testing, and cross-validation studies are ongoing. If it can be safely assumed that all RT-QuIC positive animals which were lost to follow-up expired over winter as a result of CWD, then the potential sensitivities of IHC and RT-QuIC using RAMALT biopsies would be 66% and 92%, respectively. Past studies have shown, however, that RAMALT still does not offer perfect sensitivity compared to postmortem testing – previously demonstrating a sensitivity of roughly 80% in elk [25,36]. This would imply that there still may be additional animals which have gone undiagnosed, as evidenced by four previously CWD negative animals which were later

harvested and found to be positive postmortem 6–8 months after sampling. The obex and retropharyngeal lymph nodes are rarely found to be infected in animals less than one year of age [52,53], suggesting that typical disease pathogenesis is greater than one year. Therefore, it seems likely that many of the animals diagnosed antemortem in year 2 may have been in preclinical stages when sampled in 2016. It is evident that more work will need to be done to estimate the true sensitivity of RAMALT and other peripheral tissues for CWD diagnosis. Importantly, it is not yet known if the advantages that RT-QuIC offers over IHC in antemortem RAMALT testing, in the forms of greater detection capability and faster turnaround time, will likewise apply to conventional postmortem testing of obex and retropharyngeal lymph nodes.

The presence of several IHC positive, RT-QuIC negative animals across both study periods is somewhat vexing. Despite historical attempts to optimize the RT-QuIC protocol with RAMALT and other tissues, further optimization may be necessary. Factors such as incomplete homogenization or other technical error, sample inhibitors, excess prion seed, and perhaps even strain variation likely impede the amplification process. While evidence for each of these factors is anecdotal, each is commonly found to adversely affect the outcome of other amplification assays like PCR.

Despite efforts to manage disease in the herd by removing infected animals, the prevalence was significantly higher in the second year of the study. While many of the animals identified as being CWD positive in year 2 were likely misdiagnosed in year 1, it is still discouraging to see the prevalence continue to climb in this herd. Test sensitivity aside, this may have been a result of several project-specific variables. First, culling has so far been limited to IHC positive cows, ignoring IHC positive bulls and animals positive by RT-QuIC only. The decision to allow IHC and RT-QuIC positive animals to be released was one made by ranch management in an effort to limit the financial impact of heavy culling. These presumed infected animals, released to intermingle with uninfected animals and die in the field, surely contribute to continued spread through contact and, eventually, contamination of the environment with their carcasses. A more aggressive culling strategy in the future, including bulls and cows positive by either IHC or RT-QuIC, may yield greater evidence of success [34]. Second, RAMALT testing may not identify animals at an early enough stage in the infection to effectively limit horizontal transmission. Previous studies have found that tonsil biopsy may allow for earlier detection [33,34,54,55], however the logistical

concerns required for rapid and efficient tonsil biopsy collections in standing or anesthetized animals were beyond the scope and abilities of this study. Perhaps most importantly, the initial prevalence in this herd was alarmingly high, much higher than the estimated prevalence elsewhere in this particular management unit [37,38], and was the driving influence for management efforts. It seems likely that transmission at this point could be sustained through environmental contamination alone; removal of infected animals may do little to stem the rising infection rates. It remains to be seen if this management approach would be more successful in newly diagnosed herds with very low prevalence. The study design for this project is a fluid one, and changes will otherwise continue to be made as more is learned about disease epidemiology and testing.

Apart from limitations in testing, the management of CWD *in situ* faces other challenges unique in some ways to CWD. Importantly, there is no available vaccine for preventing the disease, nor is one on the horizon. Several groups have reported pilot studies on vaccination candidates, however to date none have proven successful enough to warrant further testing [56–58]. Herd depopulation or reduction may be successful in the short term, however neither are long-term solutions for the global health of cervid populations. Reduced susceptibility to infection, as has been found in the present study, as well as other models of TSEs [59–63], is a tempting avenue of pursuit that should be thoroughly vetted. Indeed, the rising frequency of the 132L allele in this herd could be considered its one important asset. The development of scrapie resistant sheep, for example, has nearly eradicated the disease from its previously endemic areas [43,45,46]. With cervids, however, resistance based on the *PRNP* allele alone is not absolute, and is better characterized as a delayed progression [18,25]. Little is known about shedding in less susceptible animals, and it is conceivable that, by outliving their peers, they may shed for longer periods. The same can be argued in vaccinated subjects, however, with precedents set for sustained shedding in subclinical, vaccinated hosts in other diseases of veterinary and human importance [64–69]. The ability for the prion agent to adapt to less susceptible animals should also be cause for concern. Again, however, agent adaptation is also commonly seen in vaccinated animals and humans [70–73], and there has been no evidence presented to date which shows that the prevalence of classical scrapie is increasing in sheep bred to be resistant [74]. Finally, resistance to CWD may not depend on *PRNP* variance alone, and the topic should

remain open for discussion, with future steps based on hypothesis-driven evidence provided by the present study and others which are ongoing.

There is, in fact, still much which can be learned from this affected herd. Microsatellite markers, which can be used to better identify animals and ascertain lineage can be useful for identifying individuals or groups of related animals which may be more or less susceptible than their peers – *PRNP* genotype notwithstanding [75,76]. It seems logical that if *PRNP* alleles conferring greater resistance are increasing, that other as yet unidentified resistance markers will be as well – markers which may be delaying CWD onset in the older 132 MM homozygous cows observed during the study, for example. It will be interesting to determine if a population inflection point can be reached, or if past models of population declines, which did not completely account for genotypic shifts, are accurate at predicting outcomes [77–80]. A pedigree developed using microsatellites may also provide greater insight into vertical transmission, from mothers (or fathers) to their offspring, similar to techniques used to characterize kuru transmission patterns [81,82]. A pedigree will likewise assist with quantifying recruitment rates to allow estimates of fitness. This information will be critical in understanding the significance of low *PRNP* allele frequencies in other populations [27,29,61,83], as well as the risk that specific individuals face for developing CWD given that parents (or other close relatives) have been infected.

The effective management of CWD in wild and farmed cervids is a challenging prospect that has so far been met with more failures than success. The limitations of currently available diagnostic tests are just one of the factors affecting management outcomes, and the present study has offered unique opportunities to investigate antemortem testing and assay sensitivity over several years. Without sustained cooperation on laboratory and field projects from the cervid farming industry, state and federal agriculture departments, and wildlife agencies, the only certainty is that CWD will continue to spread on both sides of the fence line.

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