EFFICACY OF ANTEMORTEM RECTAL BIOPSIES TO DIAGNOSE AND ESTIMATE PREVALENCE OF CHRONIC WASTING DISEASE IN FREE-RANGING COW ELK (CERVUS ELAPHUS NELSONI)

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ABSTRACT: A reliable antemortem test is needed to understand the ecology of chronic wasting disease (CWD) in elk (Cervus elaphus nelsoni). We measured the ability of antemortem biopsy samples from the rectal mucosa to detect the abnormal prion protein associated with CWD (PrP\textsuperscript{CWD}), the relationship between test results from the obex and rectal biopsies at varying stages of CWD progression, and the prevalence of CWD in free-ranging elk from Rocky Mountain National Park, Colorado, USA. We sampled and placed radio collars on 136 adult female elk in the winter of 2007–08. Elk with biopsy samples found positive for PrP\textsuperscript{CWD} by immunohistochemistry (IHC) were euthanized and the obex and retropharyngeal lymph nodes were examined with IHC. We resampled, euthanized, and necropsied 20, 25, and 34 of the remaining study elk in each of the three following winters, respectively. Sensitivity of rectal biopsy samples increased in an asymptotic fashion with follicle count and was maximized at 85\% (95\% credible limits [CL] 5 60, 98) in the beginning of the study, when a greater proportion of elk were in a detectable stage of prion infection. However, maximum sensitivity was reduced to 72\% (CL 5 46, 94) when we included resampled elk, which included recently infected elk that were initially negative using rectal biopsies and IHC. Test results were similar between rectal biopsies and the obex, but the earliest stages of prion infection were only detected by using retropharyngeal lymph nodes. Minimum CWD prevalence was estimated to be 9.9\% (CL 5 5.7, 15.7) using rectal biopsies, but this rose to 12.9\% (CL 5 8.0, 19.1) when we included four elk that were likely misdiagnosed at initial capture. Our results indicate rectal biopsies can provide a useful research tool for CWD in elk populations, but should be used with caution because they can miss individuals in early stages of infection and underestimate prevalence. Prevalence estimates from this population are the highest reported to date in elk and indicate that under appropriate conditions, CWD may be able to affect the dynamics of high-density elk populations.

Key words: Biopsy, Cervus elaphus, chronic wasting disease, Colorado, CWD, elk, prion, rectal mucosa.

INTRODUCTION

The ability to accurately diagnose the infection status of free-ranging animals is essential to understanding the effects of a wildlife disease. Such efforts can be particularly challenging in the case of new or emerging diseases, which may lack accurate diagnostic tests or knowledge on pathogenesis and transmission mechanisms (e.g., Daszak et al., 2004). Molecular diagnostics can overcome many of these issues (Tang et al., 1997), but significant obstacles remain for relatively novel infectious agents that do not have nucleic acids, occur primarily in localized tissues or organs, or have multiple routes of infection. Chronic wasting disease (CWD), a transmissible spongiform encephalopathy that occurs in several wild cervid species of North America, exemplifies many of these challenges.

A reliable antemortem test is needed to better understand the ecology of CWD in
elk (*Cervus elaphus nelsoni*), and is required to estimate prevalence in herds that occur in areas without hunting or culling programs. Abnormal prion proteins that cause CWD in deer (*Odocoileus* spp.) and elk (*PrP<sub>CWD</sub>*) accumulate in neural and lymphoid tissues (Schreuder et al., 1998; O’Rourke et al., 2000; Spraker et al., 2002; Wild et al., 2002; Williams, 2005). Immunohistochemistry (IHC) assays of biopsy samples from the palatine tonsil and rectal mucosa of deer are accurate preclinical antemortem tests for *PrP<sub>CWD</sub>* (Wild et al., 2002; Wolfe et al., 2007; Keane et al., 2008, 2009), but there is comparatively limited information on the use of these techniques in elk. In comparisons of lymphoid tissues from 308 captive elk, postmortem samples of rectal mucosa accurately diagnosed six of seven infected elk (Spraker et al., 2006). Similarly, in comparisons among 17 captive elk, antemortem rectal biopsies accurately diagnosed six of six infected samples, five in the preclinical stages of disease. Additional biopsies were negative, but no postmortem samples were taken to assess sensitivity (Spraker et al., 2009b). These findings support the use of this technique to detect *PrP<sub>CWD</sub>* in elk, but measures of accuracy are needed to determine test limits (Spraker et al., 2006, 2009b).

To improve the ability to diagnose CWD and estimate prevalence, we studied the efficacy of rectal biopsies in a live-animal test for *PrP<sub>CWD</sub>* in free-ranging elk. Our specific objectives were to 1) quantify the influence of follicle counts on sensitivity and specificity of antemortem biopsies from the rectal mucosa to detect *PrP<sub>CWD</sub>*; 2) describe the relationship between *PrP* genotype and diagnostic test results using brainstem and rectal biopsies at varying stages of CWD progression, and 3) estimate prevalence of CWD in adult female elk from Rocky Mountain National Park (RMNP).

**MATERIALS AND METHODS**

**Study area and population**

This study was conducted on the elk winter range in RMNP, Colorado (40°36’N, 105°58’W), which ranges from 2,400 m to 2,800 m in elevation and consists of open meadows and riparian willow habitat that are bordered by lodgepole pine (*Pinus contorta*) and Douglas-fir (*Pseudotsuga menziesii*) on north-facing slopes and ponderosa pine (*Pinus ponderosa*) and upland shrub communities on south-facing slopes. Elk winter range in the park encompasses approximately 10,000 ha. Density estimates for this area were 15–110 elk/km<sup>2</sup> during 1995–2000 (Singer et al., 2002).

Chronic wasting disease has been present in RMNP for over 30 yr (Spraker et al., 1997). Prevalence of CWD has not been previously estimated from elk that inhabit RMNP. Prevalence in harvested elk in adjacent areas outside of RMNP was estimated to be 2.4% (nine of 382) from 2006–08 (Colorado Division of Wildlife, 2009). Prevalence of CWD in mule deer (*Odocoileus hemionus*) from areas adjacent to the elk winter range in RMNP (in and around the town of Estes Park) was 8.3% (15 of 181) in 2002–03 (Wolfe et al., 2004).

**Study design**

Adult female elk were initially captured, sampled, and released with a radio collar during the winter of 2007–08 (*n* = 136; hereafter referred to as initial captures). Thirteen of the initial captures were positive for *PrP<sub>CWD</sub>* (CWD+) via IHC of rectal biopsies and were recaptured, euthanized, and necropsied within 55 days of initial capture. We then resampled, euthanized, and necropsied 20, 25, and 34 of the remaining study elk in each of the three following winters, respectively (hereafter referred to as resampled elk). The status of each study elk (alive or dead) was monitored at least once per week using radiotelemetry. Whole carcasses were necropsied in the field or lab to determine cause of death if elk died during the study. Postmortem samples from the brainstem (medulla oblongata at the level of the obex; obex hereafter) and retropharyngeal lymph nodes were collected during all necropsies for CWD diagnostics.

**Elk capture and antemortem samples**

We anesthetized adult female elk using 2.7–3.3 mg of carfentanil (Wildlife Pharmaceuticals, Fort Collins, Colorado, USA) and 10 mg of xylazine (Tranquived, Vedco, St. Joseph,
Inject samples were assayed for PrP neutral buffered formalin. Lymph nodes were removed and stored in 10% neutral buffered formalin. The obex and medial retropharyngeal lymph nodes were considered PrP positive. Obex samples that contained coarse, red, granular, or particulate deposits surrounding neurons and scattered in the neuropil were considered CWD+. Obex samples were scored on a scale from 0 (no PrP \text{CWD} detected) to 10 (heavy accumulation of PrP \text{CWD}) as previously described (Spraker et al., 2010). Lymph nodes and rectal mucosa were considered CWD+ if follicles or nerves had coarse, bright red granular material (Spraker et al., 2009b). Elk without IHC staining were considered uninfected (CWD−). For each rectal biopsy, we quantified the number of follicles with and without IHC staining (Spraker et al., 2006).

Analyses

We modeled the proportion of animals correctly diagnosed as CWD+ (sensitivity) as follows. False negatives were caused by observing 0 positive follicles in an animal that was infected. There were two sources of such zeros. The first source was due to sampling a finite number of follicles in elk that have PrP \text{CWD} in the rectal tissue. There was almost always a chance of observing 0 positive follicles when the number of follicles in a sample was small because the percentage of positive follicles generally ranges from 20% to 80% in rectal biopsies from elk (Spraker et al., 2006, 2009b). The second source of zeros was too little or no PrP \text{CWD} for IHC to detect in the rectal mucosa of a recently infected elk. In this case, elk may have been infected, but because the infection was recent, PrP \text{CWD} may not have sufficiently accumulated in the rectal mucosa to be detected with IHC.

To deal with these two sources of zeros, we modeled detection probability using a hierarchical, zero-inflated binomial model:

\[
\Pr(p,z,\pi,a,b|y,n) = \prod_{i=1}^{N} \operatorname{binomial}(y_i|p_i(1-z_i),n_i) \times \operatorname{Bernoulli}(z_i|\pi) \times \operatorname{beta}(p_i|a,b) \times \operatorname{gamma}(a|0.001,0.001) \times \operatorname{gamma}(b|0.001,0.001)|\beta(\pi|1,1) \]

where \(N\) is the number of animals sampled; \(p_i\) is the probability that a single follicle drawn from animal \(i\) is positive, conditional on prions being present in rectal tissue; \(y_i\) is the number of positive follicles; and \(n_i\) is the number of follicles in a sample. The parameter \(\pi\) is the probability that the rectal tissue of an infected animal does not contain enough prions for diagnostic purposes. We modeled \(p_i\) as a

Postmortem samples and diagnostic procedures

Euthanized elk were transported intact to the Veterinary Diagnostic Laboratory at Colorado State University (Fort Collins, Colorado, USA). Necropsies were conducted 2–8 hr after death. The obex and medial retropharyngeal lymph nodes were removed and stored in 10% neutral buffered formalin.

Antemortem rectal biopsies and postmortem samples were assayed for PrP \text{CWD} by IHC (Spraker et al., 2006). We considered IHC staining of any postmortem sample (obex or retropharyngeal lymph node) to be a definitive diagnosis of infection with PrP \text{CWD}. Elk with IHC positive staining were considered PrP \text{CWD} positive.
random draw from a beta distribution, treating it as a random effect that varied among individual animals. Posterior predictive checks (Gelman et al., 2004; Gelman and Hill, 2009) revealed a simple model with a single value for $p$ failed to adequately represent the variation in the data. However, the hierarchic model showed no evidence of lack of fit (Bayesian $P$ for mean $=0.74$, for standard deviation $=0.35$). The mean of the distribution of $p_i$ was estimated as $a/\pi b$ and sensitivity ($P_d$) was estimated as a function of the number of follicles in a sample ($n$) using the following equation:

$$P_d = \left(1 - \text{binomial}\left(0, \frac{a}{a+b}, n\right)\right) (1 - \pi) \quad (2)$$

where the posterior distribution of $P_d$ was obtained for a range of values of $n$ by calculating $P_d$ as a function of $a$, $b$, $\pi$, and $n$ at each iteration in a Monte Carlo Markov chain.

Analyses were conducted with JAGS 3.1.0 (Plummer, 2003, 2011a) using the rjags package (Plummer, 2011b) in the R 2.15.1 computing environment (R Development Core Team, 2012). Three chains were computed for each parameter with initial values chosen to be diffuse relative to posterior distributions (Brooks and Gelman, 1998). After discarding the first 5,000 iterations, we accumulated 30,000 samples from each chain. Convergence was assured by visual inspection of trace plots to assure stationarity and homogeneous mixing, and by the diagnostics of Gelman (Brooks and Gelman, 1998) and Heidelberger (Heidelberger and Welch, 1983) implemented in the coda package (Plummer et al., 2010) in R.

We estimated the effect of the parameter $\pi$ on sensitivity in three ways. First, we assumed all infected elk had sufficient amounts of $\PrP^{\text{CWD}}$ in rectal mucosa for diagnostic purposes, and that sensitivity was entirely reliant on obtaining enough follicles ($\pi=0$ in Equation 2). Next we estimated parameters in Equation 1 using data that only included false negatives observed at the beginning of the study. Elk were considered to be a false negative at initial capture if they were classified as CWD$^-$ by their initial rectal biopsy and died of CWD within 24 mo (Williams et al., 2002; Hamir et al., 2006). Finally, we estimated the parameters in Equation 1 using data ($y$, $n$) that included false negatives during the entire 3-yr study. Resampled elk were considered to be false negatives if they were classified as CWD$^-$ by the rectal biopsy taken during resampling efforts, but subsequently found to be CWD$^+$ at postmortem. We distinguished between estimates of sensitivity made at the start of the study versus those made over the entire study because the parameter $\pi$ depends on the extent of infection in elk (Spraker et al., 2006, 2009b). The removal of all elk known to be infected at the start of the study resulted in a study population of elk that were either not infected or too early in the course of infection to detect $\PrP^{\text{CWD}}$. Thus, we expected sensitivity to be higher at initial capture when compared to estimates that included resampled elk.

We quantified the relationship between detection of $\PrP^{\text{CWD}}$ in a rectal biopsy and disease progression by comparing obex scores among three CWD$^+$ diagnostic categories: 1) elk that had $\PrP^{\text{CWD}}$ in at least one postmortem sample (the obex or a retropharyngeal lymph node), but did not have $\PrP^{\text{CWD}}$ in their most recent rectal biopsy; 2) elk that were euthanized and had $\PrP^{\text{CWD}}$ in both a postmortem sample and their rectal biopsy; and 3) elk that died of CWD and had $\PrP^{\text{CWD}}$ in at least one postmortem sample. For the first two categories and genotype comparisons, we only included elk that had a rectal biopsy within 55 days of euthanasia. We assumed elk died of CWD when there were no other causes of death found in the field or lab and postmortem tests confirmed the presence of $\PrP^{\text{CWD}}$ in retropharyngeal lymphoid tissues and the brainstem with an obex score $\geq 8$.

We compared antemortem rectal biopsy results to definitive diagnostic tissues to estimate the proportion of animals correctly diagnosed as CWD$^-$ (specificity) using the conjugate relationship for a binomial distribution with an uninformative beta prior. All probabilities were multiplied by 100 and reported as a percentage.

**RESULTS**

Rectal biopsies indicated 13 of 136 elk were CWD$^+$ during initial capture efforts (December 2007–March 2008), all of which were euthanized and confirmed to be infected via postmortem sampling. Nine elk died of CWD during the study; four of these were found dead within 24 mo of initial capture and assumed to be misdiagnosed at the start of the study, and the other five died 25–32 mo after initial capture. We obtained postmortem samples from these animals but did not resample them before death. In addition,
one elk harvested 12 mo after initial capture was CWD+. Among resampled elk, we found nine of 79 were CWD+, but we did not detect PrP\textsuperscript{CWD} in rectal biopsies taken from six of the infected elk. Obex samples also failed to detect PrP\textsuperscript{CWD} in five of the six resampled elk that rectal biopsies misdiagnosed; these infections were only detected in the retropharyngeal lymph nodes. The one case where obex and rectal biopsy results differed was the only instance where PrP\textsuperscript{CWD} was not detected in the retropharyngeal lymph nodes, but was detected in the obex. Overall, rectal biopsies correctly identified 11 of 12 (92\%) CWD+ elk with the genotype MM\textsubscript{132}, and five of 10 (50\%) CWD+ elk with the genotype ML\textsubscript{132}. The genotype LL\textsubscript{132} was detected in our study population, but none were diagnosed with PrP\textsuperscript{CWD}.

Sensitivity increased with follicle count and ranged from 24\% (95\% credible limit [CL]=24, 58) with one follicle to over 95\% (CL=81, 99) with six follicles, when we assumed PrP\textsuperscript{CWD} was always present in the rectal mucosa of CWD+ elk (Fig. 1A). However, when we included the probability that the rectal tissue may not contain enough prions for diagnostic purposes, maximum sensitivity was 85\% (60, 98) at initial capture and 72\% (46, 94) during the study (Fig. 1B, C). As in past studies (Spraker et al., 2006, 2009b), no CWD− elk were incorrectly identified as CWD+ using rectal biopsies and IHC (specificity CL=80, 100).

Obex scores differed among the diagnostic categories of CWD+ elk detected during initial and resample efforts: elk that had PrP\textsuperscript{CWD} in at least one postmortem sample, but did not have PrP\textsuperscript{CWD} in a rectal biopsy, had a median obex score of 0.0 (CL=0.0, 0.61; n=6); elk that had PrP\textsuperscript{CWD} in their rectal biopsy had a median obex score of 6.5 (CL=4.0, 6.6; n=16); and elk that died of CWD had a median obex score of 10.0 (CL=9.1, 10.0; n=9). Obex scores differed little between PrP genotypes; elk with the genotype MM\textsubscript{132} had a median obex score of 6.2
Based on the initial rectal biopsies and postmortem samples, minimum prevalence in 2008 was 9.9% (CL = 5.7, 15.7; 13 of 136 elk were CWD+). When we included the four elk that were considered misdiagnosed during initial sampling efforts, the prevalence estimate increased to 12.9% (CL = 8.0, 19.1). Subsequent recapture and euthanasia efforts over 3 yr found that 12.0% (CL = 6.2, 20.3; nine of 79 elk) of resampled elk were CWD+ based on postmortem samples. Overall, 32 of 116 elk (27.8%; CL = 20.2, 36.4) were found to be CWD+ during the course of the 3-yr study. This estimate only includes elk that had a definitive diagnosis made with postmortem samples, and does not include 15 elk that were still alive at the end of the study and five elk that had no testable tissue when recovered.

DISCUSSION

Rectal biopsies from elk provide a useful monitoring and research tool for CWD, particularly for herds where the disease is already established. This conclusion is supported by several findings in this study; observed specificity was high, rectal biopsies and obex samples produced similar results, and the only misdiagnoses occurred in elk that were in early stages of infection. Previous work also found obex and rectal mucosa samples to be similar, with 13/14 captive CWD+ elk having the same diagnosis in both tissues (Spraker et al., 2006, 2009b). As in this study, the one exception was an elk that had minimal accumulation of PrP<sup>CWD</sup> around several neurons in the obex, but no PrP<sup>CWD</sup> in a rectal biopsy (Spraker et al., 2009b; see also Spraker et al., 2004). These results suggest PrP<sup>CWD</sup> occurs in the obex prior to the rectal mucosa. It is unknown how long it takes for this to occur, but it appears that CWD can be detected in a rectal biopsy shortly after PrP<sup>CWD</sup> accumulates on both the periphery of the neurons and within the surrounding neuropil of the obex (Spraker, unpubl. data). This is consistent with the finding that sensitivity is similar when using samples from the obex (80%; Spraker et al., 2004) and the rectal mucosa (~72–85%; observed in this study) of infected elk.

Misdiagnosis of early cases of prion infection using rectal biopsies has also been observed in white-tailed deer (<i>Odocoileus virginianus</i>; Keane et al., 2008, 2009). As in other studies (e.g., Hibler et al., 2003; Spraker et al., 2004), we found retropharyngeal lymph nodes were the most accurate diagnostic tissue in elk.

There are clear limitations to using rectal mucosa as a diagnostic tissue for detection of PrP<sup>CWD</sup> in elk, which has important consequences for captive and free-ranging herd management. Rectal biopsies misdiagnose some infected elk (at least 15–28% in this study), the rate of which will depend on individual- (PrP genotype, extent of infection) and population-level factors (prevalence, history of exposure) at the time of sampling. For example, infected elk with the genotype MM<sub>132</sub> were correctly diagnosed almost twice as often as elk with the ML<sub>132</sub> genotype. Prior research indicates MM<sub>132</sub> elk exhibit shorter incubation times for CWD than those with ML<sub>132</sub> (Hamir et al., 2006), which likely results in faster accumulation of PrP<sup>CWD</sup> in the rectal mucosa and a greater likelihood of accurate prion diagnosis using rectal biopsies (see also Keane et al., 2008, 2009). Additionally, if a herd has only recently been exposed to CWD, most infections will be recent, prevalence will be relatively low, and PrP<sup>CWD</sup> could be less detectable than in our study herd. These results indicate that IHC of rectal biopsies can underestimate prevalence, will be least useful in free-ranging populations when the prevalence of CWD is low (e.g., 1–3%), and should not be used to infer the absence of CWD during relocation efforts. However,
because the test appears to have high specificity, it can be used in conjunction with other surveillance methods or for intensive test-and-remove management strategies (e.g., Wolfe et al., 2004). Rectal biopsies will be particularly useful for such efforts if it is found that prions are primarily shed or elk are long-lived after infection of the rectum occurs and PrP\textsuperscript{CWD} can be detected.

We found that sensitivity increased with follicle count, particularly when there were fewer than five follicles (Fig. 1). It has been tentatively suggested that at least 10 follicles are needed to obtain accurate diagnostic results from a rectal biopsy (Spraker et al., 2009a, b). Because follicle counts decline with age, most samples from elk that are older than 8 yr cannot meet this requirement (Spraker et al., 2009a). We suggest that for population-level inferences there is no need to obtain a minimum number of follicles because modern methods of hierarchic modeling (Clark and Gelfand, 2006) allow uncertainty associated with diagnosis to be included in a statistically appropriate way to estimate quantities of interest such as prevalence (see also Wolfe et al., 2002). At the individual level, our results indicate that the largest declines in sensitivity occur when there are five or fewer follicles (Fig. 1). The presence of IHC staining on only the nerves in some rectal biopsy samples also suggests the presence of PrP\textsuperscript{CWD} in the rectal mucosa, and not the number of follicles, may be a limiting factor when using rectal mucosa samples to diagnose prion infection.

The minimum prevalence of CWD (9.9%) observed during our initial capture efforts is the highest estimate for a free-ranging elk population published to date. Several findings indicate that true prevalence is likely higher in this population. First, resampling and necropsy efforts confirmed that rectal biopsies misdiagnosed infected elk that were in an early stage of infection. Second, sensitivity was estimated to be as low as 72% (CL=46, 94). Finally, despite the removal of all elk found to be CWD+ via rectal biopsies, recapture efforts over 3 yr found that 12.0% of resampled elk were CWD+. These results indicate that prevalence of CWD in 2008 was likely closer to the estimate of 12.9%, which includes the four elk that were considered misdiagnosed at initial capture. The observed prevalence in this study may be limited to our study population or a relatively small region around it, as harvested elk throughout Colorado exhibited prevalence values <2.5% from 2006 to 2008 (Colorado Division of Wildlife, 2009).

In mule deer, prevalence of CWD can exceed 20% prevalence in adult females, increase mortality rates, and contribute to lower population growth rates (Miller et al., 2008). Sargeant et al. (2011) found CWD mortality, mountain lion (Puma concolor) predation, and reduced recruitment stabilized elk population growth at Wind Cave National Park during the last 10 yr (finite rate of increase=1.0, prevalence unknown). It is unknown if CWD has the potential to reach prevalences found in mule deer or substantially increase the annual mortality of elk. However, the prevalence of CWD in our study population is disconcerting because more than a quarter of the adult female elk sampled were infected with PrP\textsuperscript{CWD} over 3 yr and elk dynamics are particularly sensitive to changes in female survival (Nelson and Peek, 1982; Raithel et al., 2007; Sargeant et al., 2011).

Our findings demonstrate that rectal biopsies can be used to provide a requisite antemortem test to better understand the effects and dynamics of CWD in elk. Similar efforts in deer have yielded significant insights into the ecology and effects of CWD (Conner and Miller, 2004; Miller et al., 2008; Dulberger et al., 2010). However, there are limitations to using rectal biopsies, for they will miss elk in the early stages of prion infection. We recommend that IHC of rectal biopsies be limited to population monitoring or research scenarios that either supplement other surveillance methods or do not
require detection of every infected elk. Finally, our results demonstrate that high-density elk populations (10–100 elk/km²) can support relatively high rates of CWD (>10% prevalence) that may substantially affect the dynamics of such populations.

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