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Detection of PrP^{CWD} in postmortem rectal lymphoid tissues in Rocky Mountain elk (*Cervus elaphus nelsoni*) infected with chronic wasting disease

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Abstract. Preclinical diagnostic tests for transmissible spongiform encephalopathies have been described for mule deer (*Odocoileus hemionus*), using biopsy tissues of palatine tonsil, and for sheep, using lymphoid tissues from palatine tonsil, third eyelid, and rectal mucosa. The utility of examining the rectal mucosal lymphoid tissues to detect chronic wasting disease (CWD) was investigated in Rocky Mountain elk (*Cervus elaphus nelsoni*), a species for which there is not a live-animal diagnostic test. Postmortem rectal mucosal sections were examined from 308 elk from two privately owned herds that were depopulated. The results of the postmortem rectal mucosal sections were compared to immunohistochemical staining of the brainstem, retropharyngeal lymph nodes, and palatine tonsil. Seven elk were found positive using the brainstem (dorsal motor nucleus of the vagus nerve), retropharyngeal lymph nodes, and palatine tonsil. Six of these elk were also found positive using postmortem rectal mucosal sections. The remaining 301 elk in which CWD-associated abnormal isoform of the prion protein (PrP^{CWD}) was not detected in the brainstem and cranial lymphoid tissues were also found to be free of PrP^{CWD} when postmortem rectal mucosal sections were examined. The use of rectal mucosal lymphoid tissues may be suitable for a live-animal diagnostic test as part of an integrated management strategy to limit CWD in elk.

Key words: Chronic wasting disease; rectal lymphoid tissues; Rocky Mountain elk.

Introduction

Chronic wasting disease (CWD), a transmissible spongiform encephalopathy, has been reported in captive and free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*).[10,11,13,17–19] Chronic wasting disease has been devastating in the captive elk industry. An estimated 12,000 to 14,000 captive elk have been killed in the western United States and western Canada in the past 5–6 years to control the disease. Several thousand free-ranging mule deer, white-tailed deer, and elk have also been killed in attempts to limit the spread of the disease in the wild. Captive elk and deer cannot be ruled out as sources of CWD for free-ranging and ranch-raised cervids; therefore, eradication of CWD in captive herds is a critical component of any strategy to control CWD. An accurate diagnostic test to identify infected elk during early preclinical stages of disease is essential for the success of this program. The objective of this study was to compare the accuracy of detecting the CWD-associated abnormal isoform of the prion protein (PrP^{CWD}) in postmortem rectal lymphoid tissue sections compared with in the brainstem, retropharyngeal lymph nodes, and palatine tonsils in Rocky Mountain elk.

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Materials and methods

A captive herd of 297 elk that had a single case of CWD 1 year previously was depopulated. Eleven elk from a small herd that was thought to be free of CWD were also killed and examined. The 308 total elk examined included 182 cows, 64 calves, and 62 bulls. Ages for the animals were recorded. The heads of these elk were brought to the Colorado State University Diagnostic Laboratory for CWD testing. The brainstem, retropharyngeal lymph nodes, and palatine tonsils were collected from each elk and immersed in 10% neutral buffered formalin. At the same time the heads were removed, the terminal 8–10 cm of rectum was also collected from each elk and taken to the Colorado State University Diagnostic Laboratory. A 1.0- to 1.5-cm strip of mucosa was removed approximately 0.5-

to 1.0-cm anterior to the mucocutaneous junction of the anus from each sample. This strip of mucosa was immersed in 10% neutral formalin.

Preserved tissues from each elk were trimmed and embedded in three paraffin blocks. The first cassette contained a cross section of obex containing the dorsal motor nucleus of the vagus nerve. The second cassette contained a single section from each of the retropharyngeal lymph nodes and one section of palatine tonsil. The third cassette contained multiple cross sections of the strip of rectal mucosa. Blocks were sectioned at 5 μm, and tissue ribbons were mounted on positively charged glass slides[a] and dried in a 65°C oven overnight. Slides were deparaffinized, placed in 99% formic acid[a] for 5 minutes at room temperature, and rinsed in running tap water for 5 minutes. Slides were then autoclaved for 20 minutes at 121°C in citrated buffer (pH 6.1),[b] cooled, and transferred into tromethamine (TRIS) buffer (APK Wash solution).[c] Staining was done with a NexES automated immunostainer at 37°C using the Basic Alkaline Phosphatase Red with Amplification Kit.[c] The primary antibody used (Anti-Prion 99)[c] was incubated for 32 minutes at 37°C. Only Amplifier A (37°C for 8 minutes) was used for staining, and Amplifier B was run using an empty dispenser. Slides were counterstained with hematoxylin.[12] A positive control slide containing brain and lymphoid tissue was stained with each run of 19 test slides. This antibody recognizes transmissible spongiform encephalopathy–positive brain in cervids, ovines, and bovines after antigen retrieval and treatment with formic acid.[12] This abnormal isoform of the prion protein has been associated with CWD of captive and free-ranging mule deer, white-tailed deer, and Rocky Mountain elk.[5,7,12–14]

A single section of brainstem at the level of the dorsal motor nucleus of the vagus nerve was scored according to the distribution of positive immunohistochemical staining (IHC).[10,14] A grade of 1 (+) was characterized by the accumulation of PrP^{CWD} in the lower half of the dorsal motor nucleus of the vagus nerve but no PrP^{CWD} in the dorsal half of the nucleus or in any of the adjacent nuclei in this single section. A grade of 2 (++) was characterized by PrP^{CWD} filling the dorsal motor nucleus of the vagus nerve but no PrP^{CWD} in any of the adjacent nuclei. A grade of 3 (+++) was characterized by PrP^{CWD} filling the dorsal motor nucleus of the vagus nerve and starting to accumulate in the adjacent nuclei. A grade of 4 (++++) was characterized by PrP^{CWD} filling the dorsal motor nucleus of the vagus nerve and an abundance of PrP^{CWD} accumulating within adjacent nuclei.

The appearance of a positive rectal mucosal lymphoid follicle was characterized by coarse, bright red, granular material within the follicle (Fig. 1). Lymphoid follicles free of IHC staining had a pale blue background (Fig. 2). Lymphoid nodes and palatine tonsils were graded on a scale of plus 1 (+) to plus 4 (++++). A plus one (+) was characterized by <10% of the follicles containing PrP^{CWD}. A plus 2 (++) was characterized by >10% but <30% of the follicles containing PrP^{CWD}. A plus 3 (+++) was characterized by >30% to <50% of the follicles containing PrP^{CWD}.

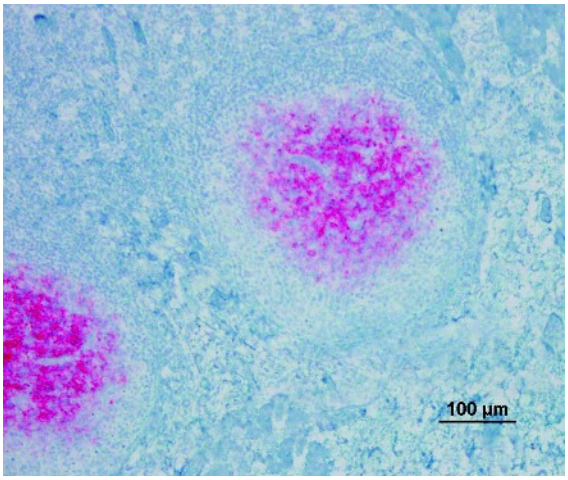

Figure 1. Photomicrograph of 2 lymphoid follicles in an elk with chronic wasting disease. Immunohistochemical staining: Streptavidin-alkaline phosphatase method. Hematoxylin/bluing counterstain. Bar = 100 μm.

A plus 4 (++++) was characterized by >50% the follicles containing PrP^{CWD}.

Follicle number and age data were analyzed to determine the relationship of the number of rectal mucosa lymphoid follicles to animal age. The pathologist designated the tissue sections as acceptable or poor. Poor biopsies meant that tissues were torn, which made an accurate count of follicles difficult. The "poor" designation did not mean there were no follicles in the tissue sections.

Animals were grouped in the following age categories: 0.5 years (calf), 1.5 years (yearling), 2.5 years, 3.5 years, 4.5 years and ≥ 5.5 years, and a mean number of follicles per age category was calculated. A linear regression was performed on log-transformed follicle numbers by age category to confirm the association noted by gross observation of the means.

Results

A total of 308 elk were examined in this investigation. Of these elk, 7 had detectable PrP^{CWD} in the dorsal motor nucleus of the vagus nerve, retropharyngeal lymph nodes, and palatine tonsil as revealed using IHC staining. None of these 7 elk showed any clinical signs of CWD before euthanasia. Six of the elk had detectable PrP^{CWD} within follicles of the rectal mucosa (Table 1).

The first elk that was positive for PrP^{CWD} was a 2.5-year-old cow. Use of IHC detected PrP^{CWD} in the dorsal motor nucleus of the vagus nerve of the brainstem, sections from both retropharyngeal lymph nodes, and a single section of palatine tonsil. The IHC section of brainstem was scored as a grade 2. On a routine hematoxylin and eosin (HE)–stained section, spongiform degeneration was not observed in the dorsal motor nucleus of the vagus nerve or in any of the adjacent nuclei. Two slides of rectal mucosa

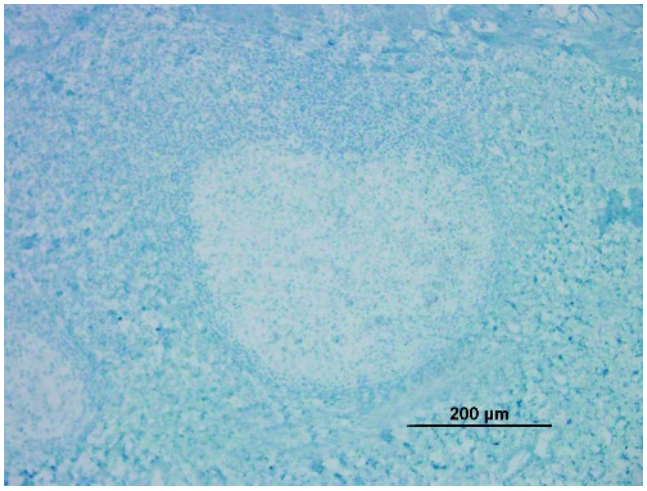

Figure 2. Photomicrograph of a lymphoid follicle in an elk free of the CWD-associated abnormal isoform of the prion protein. Immunohistochemical staining: Streptavidin-alkaline phosphatase method. Hematoxylin/bluing counterstain. Bar = 200 µm.

were examined: 167 follicles were found and 122 (73%) contained detectable PrP^{CWD}.

The second elk that was positive for PrP^{CWD} was a 7.5-year-old cow. Using IHC, PrP^{CWD} was detected in the brainstem, sections from both retropharyngeal lymph nodes, and a single section of tonsil. The IHC section of brainstem was scored as a grade 3. On a routine HE-stained section, spongiform degeneration was observed in the dorsal motor nucleus of the vagus nerve but not in any of the adjacent nuclei. Two slides were examined from the rectal mucosa: 29 follicles were found and 20 (69%) contained detectable PrP^{CWD}.

The third elk that was positive for PrP^{CWD} was a 2.5-year-old cow. PrP^{CWD} was detected in the brainstem, sections from both retropharyngeal lymph nodes, and a single section of tonsil. The IHC section of brainstem was scored as a grade 2. On a routine HE-stained section, spongiform degeneration was not observed in the dorsal motor nucleus of the vagus nerve or in any of the adjacent nuclei. One slide was examined from the rectal mucosa: 163 follicles were found and 85 (52%) contained detectable PrP^{CWD}.

The fourth elk that was positive for PrP^{CWD} was a 2.5-year-old cow. IHC documented PrP^{CWD} in the brainstem, sections from both retropharyngeal lymph nodes, and a single section of tonsil. The IHC section of brainstem was scored as a grade 3. On a routine HE-stained section, spongiform degeneration was observed in the dorsal motor nucleus of the vagus nucleus but not in any of the adjacent nuclei. One slide was examined from the rectal mucosa: 10 follicles were found and 8 (80%) contained detectable PrP^{CWD}.

The fifth elk that was positive for PrP^{CWD} was a 1.5-year-old bull. Use of IHC demonstrated PrP^{CWD} in the brainstem, sections from both retropharyngeal lymph nodes, and a single section from the tonsil. The IHC section of brainstem was scored as a grade 3. On a routine HE-stained section, spongiform degeneration was observed in the dorsal motor nucleus of the vagus nerve but not in any of the adjacent nuclei. One slide was examined from the rectal mucosa: 90 follicles were found and 56 (62%) contained detectable PrP^{CWD}.

The sixth elk that was positive for PrP^{CWD} was a 6.5-year-old bull. IHC documented PrP^{CWD} in the brainstem, sections from both retropharyngeal lymph nodes, and a single section from the tonsil. The IHC section of brainstem was scored as a grade 3. On a routine HE-stained section, spongiform degeneration was observed in the dorsal motor nucleus of the vagus nerve but not in any of the adjacent nuclei. One slide was examined from the rectal mucosa: 12 follicles were found and 6 (50%) contained detectable PrP^{CWD}.

The seventh elk that was positive for PrP^{CWD} was a 4.5-year-old bull. PrP^{CWD} was observed in the brainstem, sections from both retropharyngeal lymph nodes, and a single section of tonsil. The IHC section of brainstem was scored as a grade 1, having only several neurons surrounded by a minimal accumulation of PrP^{CWD} in each side of the dorsal motor nucleus of the vagus nerve; PrP^{CWD} was not found in any of the adjacent nuclei of the brainstem. On a routine HE-stained section, spongiform degeneration was not observed in the dorsal motor nucleus of the vagus nerve or in any of the adjacent nuclei. Three slides of the postmortem rectal mucosal tissues were examined from this elk: 45 follicles were found and all were free of detectable PrP^{CWD}.

A single slide of postmortem rectal mucosa from each of the remaining elk in which PrP^{CWD} was not detected in the brainstem or cranial lymphoid tissues was examined for evidence of accumulation of PrP^{CWD}. Rectal lymphoid follicles were found in 300 of these 301 elk, but PrP^{CWD} was not detected in the rectal lymphoid follicles in any of these animals.

Of the 308 slides of rectal mucosa obtained, the pathologist described 15 as poor tissue sections, and they were not used in the analysis. The rectal tissue sections from the remaining 293 animals were grouped by age category and the mean number of rectal mucosal lymphoid follicles was calculated. The mean number of rectal mucosal lymphoid follicles decreased with each age category indicating a possible association between follicle numbers and animal age

Table 1. Summary of age, sex, and immunohistochemical staining of the brainstem, retropharyngeal nodes, and palatine tonsil compared to postmortem rectal lymphoid tissues in the 7 elk positive for the CWD-associated abnormal isoform of the prion protein.

Elk No.	Age (yr)	Sex	Grade brain	Retro-pharyngeal node	Palatine tonsil	Rectal tissue: No. of follicles positive/ No. of follicles found (%)
1	2.5	F	2	++	++	122/167 (73%)
2	8.5	F	3	++	++	20/29 (69%)
3	2.5	F	2	++	++	85/163 (52%)
4	2.5	F	3	++	++	8/10 (80%)
5	1.5	M	3	++	++	56/90 (62%)
6	6.5	M	3	++	++	6/12 (50%)
7	4.5	M	1	+	+	0/45 (0%)

(Table 2). A significant linear regression relationship was demonstrated between age category and number of follicles ($P \leq 0.0001$) confirming this relationship.

Discussion

Early accumulation of PrP^{CWD} in tonsil and retropharyngeal lymph nodes is a reliable marker for preclinical postmortem[4,5,10–14] and antemortem[16] diagnosis of CWD in deer. Experimental oral infection of mule deer resulted in PrP^{CWD} accumulation in alimentary tract–associated lymphoid tissues (retropharyngeal lymph node, tonsil, Peyer's patches, mesenteric and ileocecocolic lymph nodes) as early as 42 days after infection.[9]

Preclinical and antemortem diagnosis of scrapie has been described in domestic sheep and is performed by biopsy of lymphoid tissue from tonsil,[8,15] nictitating membrane,[6] and rectal mucosa.[1–3] The disease-associated isoform of the ovine prion protein is detectable in lymphoid tissue at approximately one-third to one-half of the incubation period in sheep, with the notable exception of sheep with the prion protein genotype VRQ/ARR, in which accumulation is limited to the central nervous system.[8] This restricted distribution is also observed in a small percentage of sheep with other susceptible genotypes (O'Rourke, personal communication, January 10, 2006).

The pathogenesis of CWD in elk is less well defined. PrP^{CWD} was detected in brain and retropharyngeal lymph nodes (68%), nodes only (19%), and brain only (13%) in a sample of 226 naturally infected farmed elk.[10] If PrP^{CWD} accumulates in the rectal mucosal lymphoid tissue at a frequency similar to that in the retropharyngeal lymph node, the relatively high percentage of CWD-infected elk with PrP^{CWD} restricted to the central nervous system will limit the sensitivity of a preclinical test for diagnosing individual animals.

Table 2. The mean number of lymphoid follicles found in postmortem sections of rectal mucosa in specific age groups from 292 farm-raised Rocky Mountain elk.

Age group in years	No. of elk	Mean No. of follicles
Calves	62	112.5
1.5	28	82.2
2.5	92	42.5
3.5	37	32.5
4.5	38	24.0
≥ 5.5	36	13.8

Another finding that would limit the use of this rectal mucosal test to identify individual animals in a herd is the results found in case 7. This 4.5-year-old bull was in an extremely early stage of CWD, having PrP^{CWD} in lymphoid tissues of the head, but only a scant accumulation of PrP^{CWD} around several neurons in the dorsal motor nucleus of the vagus nerve. Using only the rectal mucosal sections, this PrP^{CWD}-positive elk would have been missed. The reason for the absence of PrP^{CWD} in the rectal lymphoid tissues was not determined, but it may suggest that lymphoid tissues of the head may become infected before lymphoid tissues in the lower gut.

Another potential limitation to this technique is the reduction in number of follicles as individual elk age. In this preliminary study, numerous lymphoid follicles were found in the rectal mucosa in elk <5 years old, but fewer follicles were found in elk >5 years old. However, the follicle number was adequate to diagnose CWD in 6 of 7 elk ages ranging from 1.5 to 8.5 years. It has been suggested that a minimum of 6 follicles should be evaluated in sheep eyelids to test scrapie.[6] The mean number of lymphoid follicles in the >5-year-old age group was 13, suggesting even this group could yield enough follicles for evaluation.

Even though this technique has some limitations, identifying infected herds by entire-herd screening may be a useful adjunct to the CWD control program by identifying positive elk before clinical signs can be detected. In this study of 308 elk, the postmortem rectal mucosal tissue sections did identify 6 of 7 (86%) positive elk with a single test. If this procedure is performed in a herd once a year for several years, it may prove to be beneficial by identifying nonclinical

CWD-positive animals, which can then be culled. This technique may also be useful for assessing the level of infection in a given herd. Perhaps these management strategies may result in limiting the spread of CWD in affected herds. This technique may have value in other uses, such as in experimental studies of CWD pathogenesis and transmission.

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