Journal of Wildlife Diseases, 48(2), 2012, pp. 425–434

DETECTION OF PrP^{CWD} IN FECES FROM NATURALLY EXPOSED ROCKY MOUNTAIN ELK (*CERVUS ELAPHUS NELSONI*) USING PROTEIN MISFOLDING CYCLIC AMPLIFICATION

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ABSTRACT: Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy affecting captive and free-ranging cervids. Currently, tests for CWD in live animals involve relatively invasive procedures to collect lymphoid tissue biopsies and examine them for CWD-associated, protease-resistant cervid prion protein (PrP^{CWD}) detected by immunohistochemistry (IHC). We adapted an ultrasensitive prion detection system, protein misfolding cyclic amplification (PMCA), to detect PrP^{CWD} in Rocky Mountain elk (*Cervus elaphus nelsoni*) feces. Our PMCA reproducibly detected a 1.2×10^{7} dilution of PrP^{CWD} (a 10% infected brain homogenate diluted 1.2×10^{6}-fold into 10% fecal homogenates), equivalent to approximately 100 pg of PrP^{CWD}/g of feces. We developed a semiquantitative scoring system based on the first PMCA round at which PrP^{CWD} was detected and fit a nonlinear regression curve to our serial dilutions to correlate PMCA scores with known PrP^{CWD} concentrations. We used this PMCA scoring system to detect PrP^{CWD} and estimate its concentration in feces from free-ranging elk from Rocky Mountain National Park, Colorado. We compared our results to PrP^{CWD} IHC of rectoanal mucosa-associated lymphoid tissue and obex from the same animals. The PMCA successfully detected PrP^{CWD} in feces from elk that were positive by IHC, with estimated prion loads from 100 to 5,000 pg PrP^{CWD}/g of feces. These data show for the first time PrP^{CWD} in feces from naturally exposed free-ranging elk and demonstrate the potential of PMCA as a new, noninvasive CWD diagnostic tool to complement IHC.

Key words: *Cervus elaphus nelsoni*, chronic wasting disease, elk, environment, feces, prion, protein misfolding cyclic amplification.

INTRODUCTION

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy affecting cervids, including mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and moose (*Alces alces shirasi*). This fatal neurodegenerative disease is caused by prions and associated with neural accumulation of PrP^{CWD}, an aberrant, pathologic form of the normal host-encoded cellular prion protein (PrP^{C}). Signs of CWD appear after a long incubation period and include weight loss, behavioral changes, and excessive salivation. Since the initial discovery of CWD in 1967 in Colorado mule deer (Williams and Young, 1980), CWD has been detected in many other states in the United States, in Canada, and in the Republic of Korea. The method of prion disease transmission between hosts remains unresolved, though prions have been found in saliva, urine, blood, feces, lymphoid tissues, milk, muscle, and antler velvet of several animal species infected with prions (Angers et al., 2006, 2009; Mathiason et al., 2006; Gonzalez-Romero et al., 2008; Maddison et al., 2009; Tamguney et al., 2009) and can remain infectious in the environment for long periods (Brown and Gajdusek, 1991; Miller et al., 2004). Determining prion loads in excreta from naturally exposed free-ranging cervids will help identify potential prion reservoirs and elucidate transmission dynamics in nature.

Currently, tests for CWD in live cervids involve concentrated acid or protease

treatment and subsequent PrP immunohistochemistry (IHC) to detect PrP^{CWD} in palatine tonsils (Wild et al., 2002; Schuler et al., 2005) of deer or rectoanal mucosa-associated lymphoid tissue (RAMALT; Spraker et al., 2006; Wolfe et al., 2007; Keane et al., 2008; Spraker et al., 2009a) of deer and elk. A less invasive antemortem diagnostic tool for CWD would be beneficial for management of free-ranging and captive cervids. However, biological fluids, fecal matter, or non–central nervous system tissues contain much less PrP^{CWD} than brain that makes detection in these samples difficult (Haley et al., 2009; Tamguney et al., 2009). Recently, a method has been devised that enables prion amplification in vitro (Saborio et al., 2001; Saa et al., 2006a). Protein misfolding cyclic amplification (PMCA) amplifies minute amounts of the protease-resistant pathological form of PrP (PrP^{res}) to detectable levels by stimulating its conversion from PrP^{C}. PMCA has been used to amplify PrP^{CWD} (Kurt et al., 2007; Meyerett et al., 2008) and detect as few as 26 hamster 263K scrapie PrP^{res} molecules in a 10^{-12} dilution of infected brain (Saa et al., 2006b), equivalent to approximately one infectious particle (Silveira et al., 2005). The unparalleled sensitivity of PMCA has resulted in successful amplification of PrP^{res} from feces of hamsters experimentally inoculated with prions (Kruger et al., 2009) and sheep naturally infected with scrapie (Terry et al., 2011). PMCA also amplified PrP^{CWD} from neural and lymphoid tissue from deer without clinical signs of CWD that were experimentally inoculated per os with feces collected from deer terminally ill with CWD (Haley et al., 2009).

We identified PMCA detection limits for PrP^{CWD} in spiked fecal samples. We also devised a PMCA scoring system to estimate PrP^{CWD} loads in feces and more objectively assess our PMCA results for diagnosing CWD in naturally exposed free-ranging elk. Unlike animals used in previous studies, these elk were potentially naturally exposed to CWD prions, rather than experimentally inoculated. Finally, we compared our PMCA results to PrP^{CWD} detection by conventional IHC on RAMALT and central nervous system tissue sections.

MATERIALS AND METHODS

Fecal samples

Test fecal samples were collected from 17 free-ranging elk from Rocky Mountain National Park (RMNP), Colorado (40°22′N, 105°36′W). Control samples were pooled from 13 captive elk from a ranch in eastern Colorado where CWD has not been detected and six captive elk from a ranch in Oregon, a state where CWD is not endemic. Control feces were collected off the ground shortly after defecation while RMNP samples were removed from the distal colon or rectum of euthanized elk during necropsy. These necropsies were performed at the Colorado State University Veterinary Diagnostic Laboratory and complied with all Institutional Animal Care and Use Committee guidelines. Feces were placed into whirl packs or sterile 50-ml conical tubes and stored at −80 C.

Feces homogenization

After thawing, 200 mg of fecal samples cut from several pellets at multiple depths were placed into a 2-ml tube containing silica beads and 1 ml of PMCA buffer #1 (150 mM NaCl, 4 mM EDTA, in phosphate-buffered saline [PBS]). Two fecal samples were prepared from each test elk. Feces were homogenized using a FastPrep machine (Thermo Scientific, Waltham, Massachusetts, USA) for 20 sec at maximum power. Following homogenization, samples were spun at 12,000 × G for 20 sec. These two steps were repeated four times. Six hundred microliters of homogenate was transferred to a 1.5-ml eppendorf tube, and mixed by pipette with 600 µl of PMCA buffer #2 (150 mM NaCl, 4 mM EDTA, 2% Triton X-100, in PBS). Samples were shaken in a heat block for 20 min (800 rpm, 37 C) then centrifuged for 5 min at 500 × G. Supernatants were stored in 1.5-ml tubes at −80 C. Multiple aliquots from the two samples from each elk were then analyzed using PMCA.

PMCA

Ten percent normal brain homogenate (NBH) was prepared in a prion-free room

from Tg5037 mice expressing cervid PrP^{C} as previously described (Meyerett et al., 2008). Twenty-five microliters of fecal homogenate was added to 25 µl of NBH in 0.2-ml tubes. Each PMCA round included control samples seeded with NBH, CWD-negative elk feces, and CWD-positive elk brain homogenate. Tubes were closed, sealed with parafilm, and suspended in a holder above a 3000MP sonicator horn (Qsonic, Newton, Connecticut, USA) filled with 180 ml of water. Samples were sonicated at 70% maximum power for 40 sec every 30 min for 24 hr at 37 C (one round). Half of each first-round sample was added to 25 µl of fresh NBH. For each subsequent round, 10 µl of sample was added to 40 µl NBH. We stopped PMCA by round nine to balance desired sensitivity (≥80%), specificity (≥90%), and expediency (≤2 wk). We typically began assaying for PrP^{CWD} by proteinase K (PK) digestion and Western blotting (WB) at round four because most fecal samples tested positive after that round.

PK digestion and WB

Samples were digested with 50 µg/ml PK (Roche, Boulder, Colorado, USA) for 30 min at 45 C. Reactions were stopped by adding lithium dodecyl sulfate sample loading buffer (Invitrogen, Madison, Wisconsin, USA) and boiling samples for 5 min at 95 C. Samples were electrophoresed, transferred, and visualized as previously described (Meyerett et al., 2008).

Clinical assessment of CWD status

Both captive and free-ranging elk were observed prior to fecal collection for signs of end-stage CWD including body condition, social isolation, lowered head or ear posture, ataxia, depression, polydipsia or polyurea, and ptyalism. If these clinical signs were observed the elk was considered to be demonstrating behavior consistent with CWD. All elk were alive and undisturbed by human approach at the time of behavior evaluation.

IHC

Obex tissue (brain stem at the level where the fourth ventricle converges into the central canal of the spinal cord and contains the dorsal motor nucleus of the vagus nerve) and RAMALT were collected from all necropsied elk and placed in 10% neutral buffered formalin and fixed for 1 wk. These tissues were processed as previously described (Spraker et al., 2009a).

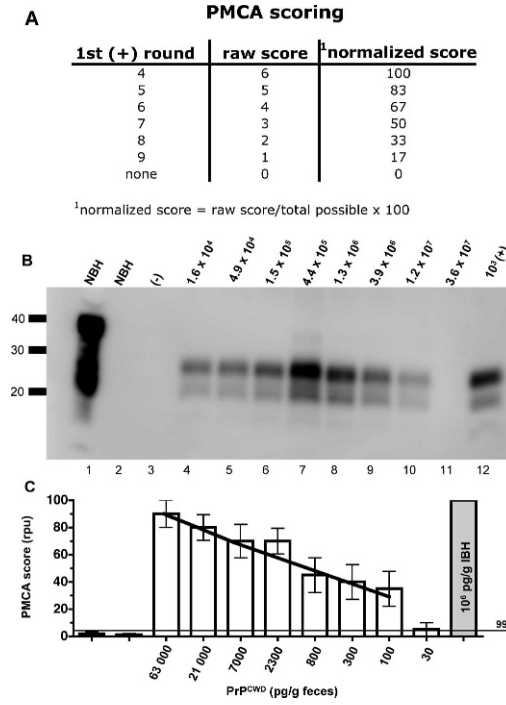

FIGURE 1. Semiquantitative protein misfolding cyclic amplification (PMCA). (A) Each elk (*Cervus elaphus nelsoni*) feces sample tested was given a score inversely proportional to the round in which it was first detected by Western blotting (WB). Samples appearing positive on an earlier PMCA round received a higher score than those appearing in later rounds. Each raw score was divided by the highest possible score then multiplied by 100 to give normalized scores. (B) Chronic wasting disease prion (PrP^{CWD}) detection limit in control feces. Samples were amplified by PMCA, digested with proteinase K (PK) except normal brain homogenate (NBH) in lane 1, then subjected to polyacrylamide gel electrophoresis (PAGE) and WB. Lanes 2 and 3 show amplified NBH and unspiked feces (negative) controls. Lanes 4–11 show amplified samples at the indicated starting dilution of CWD-positive brain spiked into control fecal homogenate. Lane 12 shows a 10^3-fold dilution of infected brain homogenate into NBH. The highest dilution reproducibly detected was $1{:}1.2\times10^7$ (lane 10). (C) PMCA scores in relative PMCA units (rpu) were plotted versus the estimated PrP^{CWD} concentration for each dilution. Nonlinear regression analysis fit a curve with the equation $y = -0.6718x^{0.3784}+17.82x^{0.1836}$ and $r^2=0.88$.

Data analyses

To quasi-quantify our PMCA results, each fecal sample was given a raw score dependent

on the PMCA round at which it first appeared PrP^{CWD} positive on a Western blot (Fig. 1A):

$$\text{Raw score} = (n+1) - r_{(+)},$$

where n = total number of PMCA rounds and $r_{(+)}$ = the first positive PMCA round.

A sample appearing positive on an earlier PMCA round received a higher raw score than those appearing in later rounds. We normalized the raw score by dividing it by the highest score possible for that sample:

$$\text{Highest possible score} = (n+1) - r_0,$$

where r_0 = the first PMCA round tested and multiplying by 100 to arrive at a PMCA score in relative PMCA units (rpu). The resulting equation is

$$\text{Normalized score (rpu)} = \left[(n+1) - r_{(+)}\right] / \left[(n+1) - r_0\right] \times 100.$$

For example, the highest possible raw and normalized score for a sample amplified for nine rounds (n=9), assayed beginning at round four (r_0=4) and found positive at round four ($r_{(+)}$=4) is 6 and 100 rpu, respectively. Samples assayed beginning at round four (r_0=4) and found PrP^{CWD} positive at round seven ($r_{(+)}$=7) received a raw score of 3 rpu and normalized score of 50 rpu. Mean PMCA scores were calculated from at least five replicate scores for each sample. A power series equation produced the best nonlinear fit of PMCA scores versus PrP^{CWD} dilutions using GraphPad software (Prism, La Jolla, California, USA). We established detection thresholds for distinguishing positive from negative PMCA samples based on the 99.9% confidence interval (CI) for designating NBH samples as negative, calculated from the mean PMCA score of 90 NBH control samples using the Student's t-table. Thus, we have reduced our chance of detecting false-positive results above this detection threshold to 0.1%. We designated samples positive only if their PMCA score rose at least 1 SEM above our detection threshold, further reducing the risk of identifying false positives.

RESULTS

Semiquantitative PMCA

To determine whether PMCA could amplify PrP^{CWD} from elk feces and, if so, the detection limit of the assay, we serially diluted brain homogenate from a CWD-positive elk into CWD-negative control elk 10% fecal homogenate and amplified samples for nine rounds of PMCA. After nine rounds of PMCA (approximately 2 wk), we achieved a specificity of 98% (two false positives of 90 NBH samples assayed, Table 1) and a sensitivity of 80% (8/10 positive samples) for our 1.2×10^7 brain dilution in fecal homogenate (Fig. 1B). Based on previous calculations of PrP^{CWD} content in the same infected brain using a standard curve generated from serial dilutions of recombinant cervid PrP^{C} (Nichols et al., 2009), we estimate that we reliably detected approximately 100 pg of PrP^{CWD}/g of feces (Fig. 1C).

We inversely correlated the PMCA score with the first PMCA round in which we detected PrP^{CWD} (Fig. 1A). Using this principle we derived a PMCA scoring system that produced a standard curve of PrP^{CWD} concentration versus PMCA score to which a nonlinear regression curve produced a very good fit (Fig. 1C, r^2=0.88). We set our PrP^{CWD} detection threshold at the 99.9% CI (3.0 rpu, based on normalized scores) for designating NBH samples negative based on their mean PMCA score (0.9±0.6). We considered samples positive if their mean PMCA scores rose at least 1 SEM above our 3.0 rpu detection threshold. Positive mean PMCA scores ranged from 90.0±10.0 rpu to 35.0±13.0 rpu for samples containing 6.3×10^4 and 100 pg PrP^{CWD}/g feces, respectively.

Determining CWD status by IHC and histopathology

We determined the CWD status of naturally exposed free-ranging elk from RMNP, a CWD-endemic area, by conventional acid hydrolysis and PrP IHC of

TABLE 1. Chronic wasting disease (CWD) status of Rocky Mountain elk (*Cervus elaphus nelsoni*) from Colorado and Oregon, USA, assessed by rectoanal mucosa-associated lymphoid tissue (RAMALT) immunohistochemistry (IHC), clinical condition, and protein misfolding cyclic amplification (PMCA) from feces.

	CWD status		
Source/location	RAMALT IHC[a]	Terminal disease	Feces PMCA[b]
RMNP[c]	20/30	No	11/14
RMNP	7/10[d]	No	2/14
RMNP	55/71	No	12/14
RMNP	1/5	Yes[e]	13/14
RMNP	15/15	No	5/10
RMNP	0/7	No	1/10
RMNP	0/0[f]	No	2/10
RMNP	0/10	No	2/10
RMNP	0/10	No	2/10
RMNP	0/10	No	1/10
RMNP	0/10	No	2/10
RMNP	0/10	No	0/10
RMNP	0/10	No	0/10
RMNP	0/10	No	0/10
RMNP	0/10	No	0/10
RMNP	0/10	No	0/10
RMNP	0/10	No	0/10
Ranch[g]	ND[h]	No	3/39
Ranch[g]	ND	No	2/47
Tg5037	ND	No	2/90

[a] Number of positive follicles/number of follicles analyzed.

[b] Number of positive samples/number of samples tested.

[c] RMNP = Rocky Mountain National Park.

[d] Only light staining evident.

[e] Only animal with clinical signs of terminal CWD.

[f] No RAMALT follicles were found.

[g] Feces from captive elk without clinical signs of CWD from ranches in Colorado ($n=13$ elk) and Oregon ($n=6$ elk).

[h] ND = not detected.

sections of obex and RAMALT removed during necropsy. Five of 17 elk tested positive for CWD by IHC. Examination of brains from four of these elk (elk 4 with clinical signs of CWD, elks 1, 3, and 5 without [Table 1]) revealed typical CWD neuropathology, including PrP^{CWD} deposition, vacuolation and microcavitation of gray matter, neuronal degeneration, and moderate astrocytosis (Fig. 2A). We also detected PrP^{CWD} in the RAMALT isolated from these elk using IHC (Fig. 2B). One additional elk (elk 2, Table 1) exhibited only pale, diffuse PrP^{CWD} staining in RAMALT follicles and minimal neuropathology in the obex. The remaining elk exhibited no PrP^{CWD} in neural tissue or RAMALT (Fig. 2C and 2D) and did not exhibit signs of end-stage CWD.

Determining CWD status by PMCA

We investigated whether PMCA could amplify PrP^{CWD} from feces collected from the same naturally exposed elk on whose neural and lymphoid tissue sections we performed IHC. PK digestion and WB of samples revealed positive PMCA results in IHC-positive animals (Fig. 3A, C). Several fecal samples from IHC-negative elk also initially tested positive (Fig. 3C). Upon retesting, five out of six samples from IHC-positive elk 3 again tested positive after six to nine rounds of PMCA (Fig. 3B and Table 1). However, the majority (110/

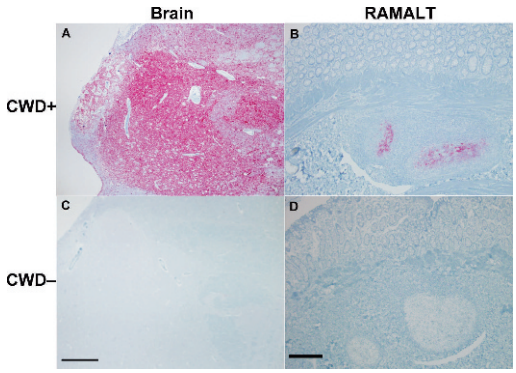

FIGURE 2. Representative chronic wasting disease prion (PrP^{CWD}) immunohistochemistry (IHC). Sections of obex, myelencephalon of the brain (A and C) and rectoanal mucosa-associated lymphoid tissue (RAMALT; B and D) from chronic wasting disease (CWD)-positive elk (A and B) display abundant, bright red chromogenic PrP^{CWD} stain in the dorsal nucleus of the vagus nerve in the brain (A) and significant stain in lymphoid follicles in RAMALT (B). IHC revealed little or no staining in sections from the CWD-negative elk (C and D). Scale bars, 50 μm for the brain and 200 μm for RAMALT.

120) of fecal samples from IHC-negative elk tested negative (Fig. 3D and Table 1), yielding a false-positive rate of 8%, the same as for control fecal samples from 13 captive Colorado elk (3/39). We observed similar false-positive rates for samples from six captive Oregon elk (4%, 2/47) and for the Tg 5037 normal brain homogenate (2%, 2/90, Table 1).

Using our PMCA scoring system we semiquantitatively assessed prion load in feces from these elk. Using 3.0 rpu as our detection threshold, PMCA detected PrP^{CWD} in feces from the elk that were clearly positive by IHC: elk 1 (45.7±14.1 rpu), elk 2 (13.33±8.3 rpu), elk 3 (56.3±9.4 rpu), elk 4 (70.6±8.7 rpu), and elk 5 (25.9±8.4 rpu, Fig. 4 and Table 1). Assuming that the CWD-infected brain we used to generate our standard curve (Fig. 1A) represents a typical prion load, and interpolating mean PMCA scores into this curve, we estimate that four of these elk shed between 100 pg and 5,000 pg of PrP^{CWD}/g feces. Since the fecal PMCA

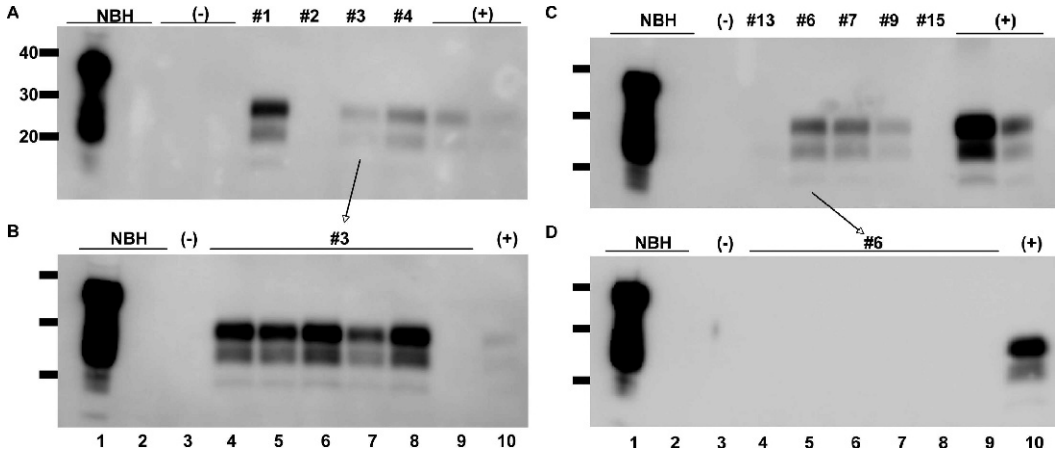

FIGURE 3. Representative feces protein misfolding cyclic amplification (PMCA). Western blots of amplified fecal samples from elk with or without clinical signs of chronic wasting disease (CWD). All samples were digested with proteinase K except for the normal brain homogenate (NBH) in lane 1. (−), negative control elk feces. (+), CWD-positive elk brain homogenate control. (A) Following seven rounds of PMCA, three of four fecal samples from immunohistochemistry (IHC)-positive elk were positive for PrP^{CWD} (lanes 5–8). (B) Further testing of fecal samples initially found positive revealed additional positive samples. Elk 3 had five of six samples positive after nine PMCA rounds (lanes 4–9). (C) A number of nonclinical, IHC-negative elk fecal samples were tested for PrP^{CWD}. In this blot, fecal samples from three of five IHC-negative elk were positive for PrP^{CWD} (lanes 4–8). (D) Additional fecal samples from these elk were tested and, as shown for elk 6 (lanes 4–9), most were PrP^{CWD} negative.

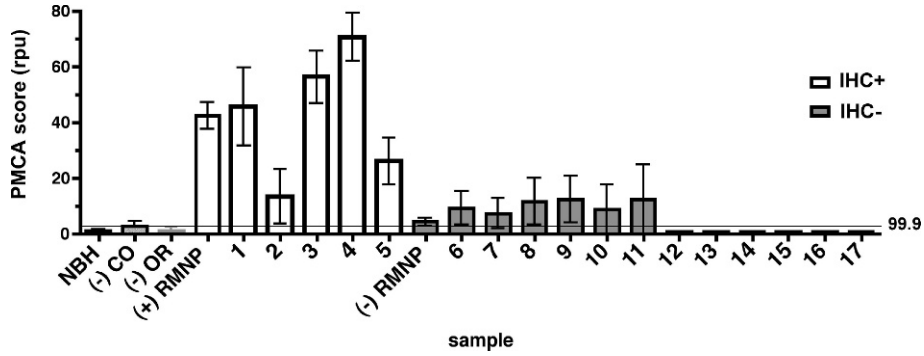

FIGURE 4. Protein misfolding cyclic amplification (PMCA) scores of elk fecal samples. Normalized mean PMCA scores for fecal samples from five immunohistochemistry (IHC)-positive elk ($n=14$, white bars) and 12 IHC-negative elk ($n=10$, black). The composite mean score is given for both groups; (+) Rocky Mountain National Park (RMNP) and (−) RMNP). Negative controls include normal brain homogenate ($n=90$) and fecal samples from 13 Colorado elk ($n=39$) and six Oregon elk ($n=47$). The horizontal line above the x-axis indicates the 99.9% confidence interval (CI).

score for elk 2 fell below our standard curve, we estimate its prion load to be <100 pg of PrP^{CWD}/g feces.

Lower limits for mean PMCA scores (i.e., mean score minus SEM) for all but one IHC-negative elk fell below the detection threshold of 3.0 rpu. We therefore designated these elk PrP^{CWD} negative and scored IHC-negative elk 9 positive by PMCA (12.5±7.8 rpu).

DISCUSSION

Several recent studies have demonstrated the presence of prions in mouse, hamster, and deer feces following oral inoculation (Maluquer de Motes et al., 2008; Safar et al., 2008; Tamguney et al., 2009). PrP^{res} had previously been amplified from feces of hamsters experimentally inoculated with prions ex situ (Kruger et al., 2009) and, more recently, from naturally infected sheep (Terry et al., 2011). The present study demonstrates for the first time the presence of PrP^{CWD} in the feces of free-ranging naturally exposed elk. Four of 17 elk tested in this study exhibited intense staining in the obex and RAMALT by IHC, with elk 3 having the most advanced case of CWD observed. Our PMCA scores correlated very well with IHC, with fecal samples from IHC-positive elk receiving the highest PMCA scores. Elk 2 appeared to be an early case of CWD, exhibiting only light PrP^{CWD} staining of RAMALT and none in the obex. PMCA detected PrP^{CWD} in only 2 of 14 samples with a correspondingly low PMCA score, also indicating a low level of infection. We also scored one IHC-negative elk positive by PMCA. We cannot determine whether PMCA detected a false positive or IHC reported a false negative because the small sample size in this study precludes definitive assessments concerning sensitivity and specificity for diagnostic purposes. However, these data do support PMCA as a prion detection assay comparable to IHC.

PMCA scoring can also be used to estimate prion load. We have found empirically that estimating PrP^{CWD} concentration from band intensities on Western blots is grossly unreliable after just one or two PMCA rounds (Pulford and Zabel, unpublished data). This subsequently hinders regression analysis to create an accurate standard curve. A more reliable means for producing such a curve relies on the more robust correlation between PrP^{CWD} concentration and the PMCA round at which it is first detected (Chen et al., 2010). Using this principle we derived a PMCA scoring system that

accurately estimated PrP^{CWD} load in spiked serial dilutions and CWD status compared to IHC.

Detection of PrP^{CWD} in cervid feces may also illuminate the contribution of fecal shedding to environmental contamination and disease transmission. For example, cervids in the vicinity of RMNP shed nearly 1 million kg of feces each year (Nichols et al., 2009). Based on CWD prevalence estimates in RMNP and the surrounding region, we estimate that 2–10% of that load contains PrP^{CWD} (Colorado Division of Wildlife, 2009), and that each gram of infected feces contains 100–5,000 pg of PrP^{CWD}, we estimate that fecal shedding may contribute 2–500 mg of PrP^{CWD} into this endemic region every year.

The PMCA protocol outlined here complements PrP^{CWD} detection by IHC in several ways. Most importantly, PMCA can detect PrP^{CWD} in easily obtainable fecal samples that are simple to process and assay, with sensitivity comparable to IHC. Fecal PMCA may also be an alternative test to IHC in older cervids that have fewer rectal lymphoid follicles present in biopsy sections (Spraker et al., 2009b). Our PMCA data suggest that fecal prion detection is independent of follicle count in RAMALT. Only one out of five RAMALT follicles from elk 4 was positive by IHC, while 13 of 14 fecal samples were positive by PMCA. Conversely, seven of 10 RAMALT follicles from elk 2 were positive by IHC, while only two of 14 fecal samples were positive by PMCA. In this case, incorporation of our scoring system, which allows for more accurate, unbiased data interpretation by transforming qualitative PMCA results to semiquantitative numerical scores and analyzing their significance statistically, enabled us to designate elk 2 positive.

Disadvantages of PrP^{CWD} detection via fecal sample PMCA include the need to maintain a colony of transgenic cervidized mice for NBH; the time, effort, and expense of nine rounds of PMCA; and, perhaps most importantly, the care needed to avoid false-positive results both during sample collection and analysis. In the field, strict attention must be given to avoid cross contamination between animals sampled. In the laboratory, specificity of PMCA can begin to wane after six rounds of PMCA without extraordinary efforts to prevent cross contamination, including a dedicated clean room for NBH preparation, compulsive glove-changing, and constant prion decontamination procedures. We found that most PMCA scores below 50 rpu required six to nine rounds of PMCA for detection, raising legitimate concern of false positives. Recently, new technical advances employ paramagnetic beads to specifically capture and concentrate PrP^{res} (Miller and Supattapone, 2011) and teflon beads added to PMCA reactions to increase efficiency two to three orders of magnitude per round (Gonzalez-Montalban et al., 2011). The latter study also noted improvements in reproducibility and specificity, due mainly to the reduced number of rounds required to detect minute quantities of PrP^{res}. We are currently testing these modifications to decrease the number of PMCA rounds, thereby improving speed, efficiency, reliability, and specificity.

ACKNOWLEDGMENTS

We thank wildlife biologists and staff at RMNP, Colorado, for their support and the National Park Service Biological Resource Management Division staff for tracking, tagging, and sampling elk. We thank Pauline Nol at US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services-Western Region, National Wildlife Research Center in Oregon for control elk feces. We thank the North American Deer Farmers Association and USDA APHIS (cooperative agreement 10-7488-0680) for funding parts of this work.

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Submitted for publication 20 March 2011.
Accepted 1 October 2011.