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Preliminary observations of genetic susceptibility of elk (*Cervus elaphus nelsoni*) to chronic wasting disease by experimental oral inoculation

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Abstract. To compare the genetic susceptibility of elk (*Cervus elaphus nelsoni*) with various alleles of the PRNP gene, which encodes the normal cellular prion protein, to chronic wasting disease (CWD), eight 8-month-old elk calves of 3 genotypes (2 132MM, 2 132LM, and 4 132LL) were orally dosed with CWD-infected brain material from elk. During postinoculation (PI) month 23, both 132MM elk had lost appetite, developed clinical signs of weight loss and central nervous system (CNS) dysfunction, and were euthanized. Two other elk (both 132LM) developed similar clinical signs of disease and were euthanized during PI month 40. All 4 affected elk had microscopic lesions of spongiform encephalopathy (SE), and PrP^{res}, the disease-associated form of the prion protein, was detected in their CNS and lymphoid tissues by use of immunohistochemical (IHC) and Western blot (WB) techniques. These findings indicate that elk with MM and LM at codon 132 are susceptible to orally inoculated CWD. All 4 LL elk are alive at PI year 4 and are clinically normal, which suggests that 132LL elk may have reduced susceptibility to oral infection with CWD-infected material or may have prolonged incubation time.

Key words: *Cervus elaphus nelsoni*; chronic wasting disease; elk; genetic susceptibility.

Chronic wasting disease (CWD) is a fatal spongiform encephalopathy, or prion disease, of cervids in North America. The natural disease has been documented in mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*O. hemionus columbianus*), white-tailed deer (*O. virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*).[12,16,17]

Elk that are homozygous for the allele encoding methionine (M) at codon 132 of PRNP (the gene encoding the normal cellular prion protein) are over-represented in free-ranging and farm-raised CWD-affected elk, compared with unaffected control groups.[7,11] Chronic wasting disease has been confirmed in elk heterozygous for 132M and the alternative allele encoding leucine (L) at codon 132. However, to the authors' knowledge, only 1 confirmed case of CWD in an elk homozygous for 132L has been reported.[11] The purpose of the study reported here was to determine the influence of codon 132 on susceptibility and incubation time in elk that had been experimentally exposed by oral inoculation of CWD-infected brain.

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Eight 8-month-old elk calves were obtained from a game farm on which captive elk were raised. The farm consisted of 3 distinct premises, and elk were moved among these for breeding, meat production, pilose antler production, and educational display. A case of CWD was confirmed on December 18, 1997, although it is probable that there were earlier undiagnosed cases. An additional 78 cases were diagnosed, the latest on March 5, 2001, when the last of the elk, white-tailed deer, and mule deer were depopulated from the 3 premises. None of the CWD-positive elk were homozygous for 132L. After the report of predisposition to disease in elk homozygous for 132M, the Elk Research Council of the North American Elk Breeders' Association and US Department of Agriculture (USDA), Agricultural Research Service (ARS) assisted the producer with a selective breeding program to develop a population of 132LL elk using 132LM and 132LL cows and 132LL bulls. Four 132LL calves from the summer 2000 birth group were moved to a quarantine facility at the National Veterinary Services Laboratories (NVSL) in Ames, IA in February and March of 2001. Two 132MM and two 132LM calves were obtained from a separate breeding group kept in 1 of the other 2 premises. Genotyping was performed as described[7] on buffy coat cells collected from live animals and on brain specimens collected at necropsy and frozen. Because of the high CWD prevalence at this facility, infection with CWD before movement to the quarantine facility cannot be ruled out.

Material for inoculation was prepared from the pooled frozen brain specimens of 2 CWD-affected

Rocky Mountain elk (one 132MM and one 132LM) from the infected herd. Both elk were adult cows that were manifesting clinical signs suggestive of CWD at the time of necropsy. Approximately 150 g of brain was mixed with 15 ml of saline and placed in sterile stomacher bags[a] and blended in ''The Stomacher.''[b] At approximately 8 months of age, each elk was given 3 ml of the mixture per os daily for 5 consecutive days. The material was aspirated into a 20-ml syringe, then was instilled in the posterior portion of the oral cavity. Material was not lost during this procedure. The pool was positive for PrP^{res}, the disease-associated form of the prion protein, by use of immunohistochemical (IHC) and Western blot (WB) analyses.

Calves were housed in a biosafety level-2 isolation barn at the NVSL, Ames, IA. Husbandry of these animals has been described.[4] Personnel wore protective clothing while in the isolation facility and showered before leaving the facility. Animals were euthanized when clinical signs suggestive of CWD (weight loss, ataxia) were observed.

Euthanasia was done by administering an overdose of pentobarbital, and a complete necropsy was conducted on each of the carcasses. The brain was cut longitudinally; one half was fixed in formalin for not less than 3 weeks, and the other half was frozen at −20°C. The formalin-fixed brain was cut into 2- to 4-mm-thick coronal sections. Sections from various anatomic sites (10 to 15 sections/animal) of cerebrum, cerebellum, and brainstem (including the medulla oblongata at the level of the obex) were processed in routine manner for histologic examination, embedded in paraffin wax, and sectioned at 5-m thickness. The sections were stained with hematoxylin and eosin (HE), and by an IHC method for detection of PrP^{res} as described.[6,11] Monoclonal antibody F99/97.6.1[5,c] was used for the detection of PrP^{res}. This antibody recognizes cervid, ovine, and bovine transmissible spongiform encephalopathy (TSE)-positive brain after antigen retrieval and treatment with formic acid.[11] In this study, CNS tissue, palatine tonsil, retropharyngeal lymph node, and gut-associated lymphoid tissue were examined.

For immunodetection of PrP^{res}, a previously described method[13] was used on frozen brain (caudal portion of the medulla). If no signal was detected using detergent extracts of the brain, the samples were enriched for PrP^{res} using phosphotungstic acid precipitation.[15] Samples were considered test positive when the 3 isoforms of the pathologic form of the prion protein, PrP^{res} were detected on the immunoblot after proteinase K enzyme treatment. A positive-control sample was prepared from the brain of a free-ranging elk (genotype 132MM) from Colorado with naturally acquired clinical disease.

By postinoculation (PI) month 23, both 132MM animals had lost their appetite, developed clinical signs of weight loss and CNS dysfunction, and were euthanized. Two other elk (both LM) developed similar clinical signs of disease and were euthanized during PI month 40. All 4 clinically ill animals had microscopic lesions of spongiform encephalopathy (SE) characterized by intraneuronal vacuolation, microcavitation of gray matter, neuronal degeneration, and mild astrocytosis, as described in deer and elk.[13,16,17] The microcavitation of gray matter was more prevalent than the intraneuronal vacuolation and neuronal degeneration (Fig. 1). The intensity of the SE within the vagus and surrounding nuclei was considered severe in all 4 elk.[11] There was no detectable difference in distribution or severity of lesions between the 132MM elk and the 132LM elk.

Abnormal prion protein was detected throughout the brain and selected lymphoid tissues of all 4 elk by use of IHC as described.[8,11,13] The appearance of PrP^{res} in the brain was a coarse red granular material, which was scattered throughout the neuropil, but was especially evident surrounding neurons. Intraneuronal staining was rare (Fig. 2). In all 4 elk, abnormal prion protein was abundant within lymphoid follicles of the palatine tonsil, retropharyngeal lymph node, and gut-associated lymphoid tissue (Peyer's patches).[11,13] There was no difference in staining intensity or distribution between the 132MM and 132LM elk.

The PrP^{res} was also detected by WB analysis in brain material from the 4 IHC-positive animals and the oral inoculum. Starting wet weight required for a detectable signal was 15 mg for the homogenized inoculum and 0.1 to 1.7 mg for caudal medulla samples. A distinct profile of the 3 isoforms of PrP^{res} (di-glycosylated, mono-glycosylated, and unglycosylated polypeptides) is shown in Fig. 3). The 5 samples had migration patterns similar to that of a reference sample prepared from the brain of a wild-caught elk with naturally acquired CWD.

Like all other TSEs, CWD is characterized by a long incubation period. Naturally affected elk manifest changes in behavior, excess salivation, and terminal emaciation.[16] In the study reported here, similar clinical signs of disease were observed in 4 elk with either 132MM or 132LM genotype. The average PI incubation period for the 132MM and 132LM elk was 22.8 and 39.2 months, respectively. Characteristic morphologic changes of SE in CNS tissues were observed in all affected elk, and were not appreciably different in the homozygous and heterozygous groups. The PrP^{res} was observed in the affected elk and was documented by use of IHC analysis of neural and lymphoid tissues and was confirmed by use of WB analysis of frozen brain.

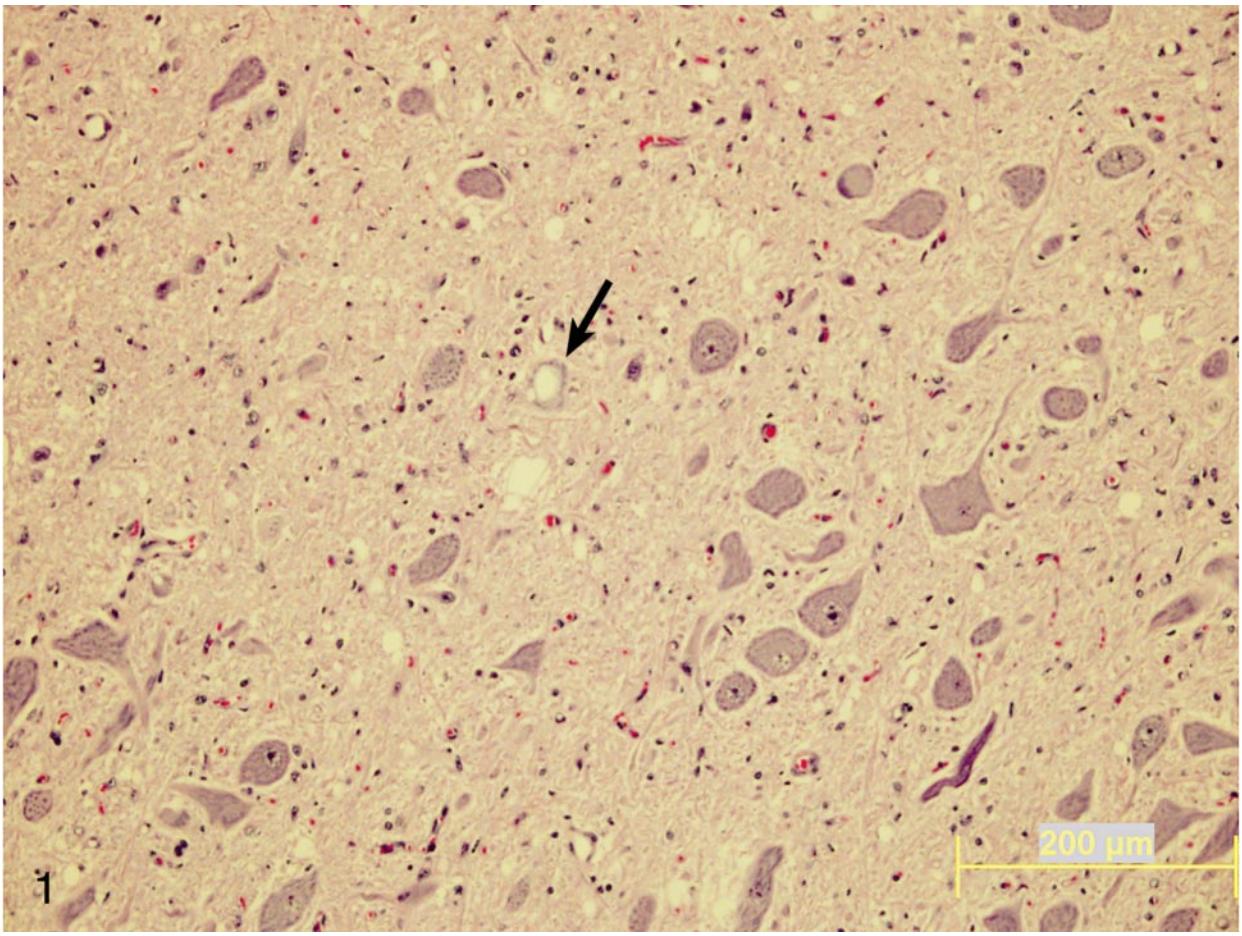

Figure 1. Brain; medulla oblongata, animal No. 1. Notice presence of severe diffuse spongiform encephalopathy characterized by clear vacuolation of neuropil and occasional presence of clear vacuole within neuronal cytoplasm (arrow). HE.

At PI year 4, the four 132LL elk are clinically normal. Because these elk were inoculated, it is certain that they will have a longer incubation period than that of the 132MM or 132LM elk. Therefore, on the basis of these preliminary observations, elk with 132MM genotype appear to have the shortest incubation period (during PI year 2). Elk with 132LM genotypes appear to be equally susceptible to the disease, but have a longer incubation period (up to PI year 3.5). Similar results were obtained when the sheep scrapie agent was inoculated into elk; the incubation period for the 132MM and 132LM elk was 3 and 4 years, respectively.[4] The incubation period for CWD in 132LL elk may be long, and the 4 surviving animals will be maintained for an additional period of 2 years (total of 6 years after inoculation). At that time, the elk will be euthanized and examined for CWD.

This study involved a small number of animals that were selected from an infected facility and exposed to CWD through experimental challenge. To further answer the issue of survival of 132LL elk exposed to CWD, larger numbers of animals, including animals sourced from CWD-free facilities, will be needed; in addition, natural cases of CWD provided by state regulatory agencies will be genotyped. To determine whether 132LL elk have the same long incubation period or low susceptibility phenotype when exposed to CWD from other species, a subsequent experiment may be necessary, in which 132LL elk would be inoculated intracerebrally with CWD-infected material from mule deer and white-tailed deer.

The association between polymorphisms in the open reading frame of the PRNP gene and susceptibility/incubation time is well documented in natural sheep scrapie, murine experimental models, and some forms of human TSE.[1,3] In addition, the association between TSE susceptibility and changes in the noncoding region of the PRNP gene or in putative accessory molecules have been suggested in several ovine, bovine, and murine studies.[2,9,10,14] The polymorphism at codon 132 was the only coding change observed in the open reading frame of the PRNP gene in the elk of this study. However, the four 132LL elk are progeny of long-term survivors in the heavily infected source

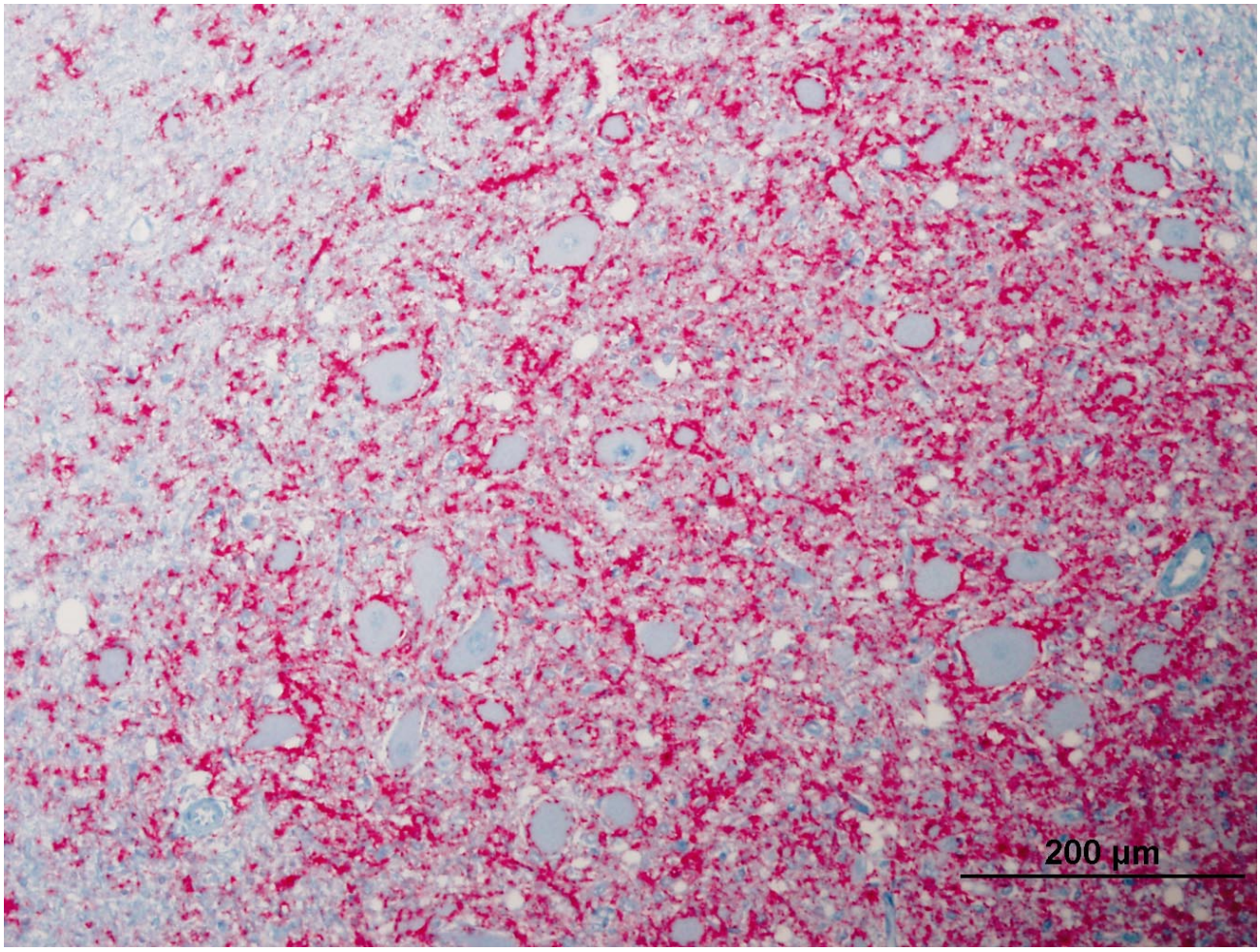

Figure 2. Brain; medulla oblongata, animal No. 1. Notice extensive presence of PrP^{res} staining (red) in the neuropil, predominantly within the neuropil and around neurons and blood vessels. Stained for PrP^{res} by IHC.

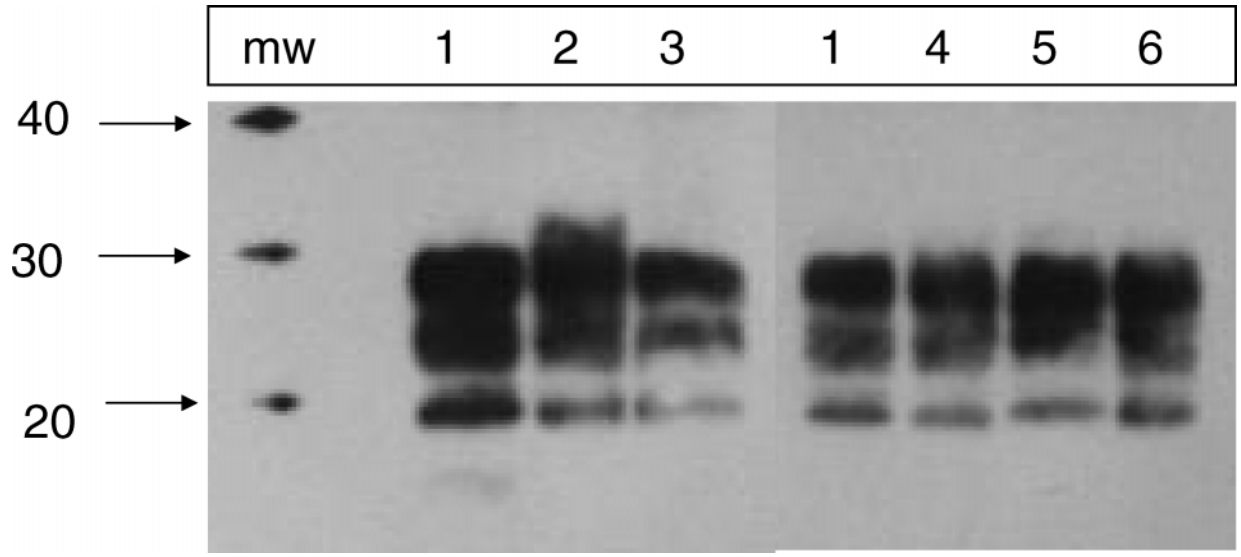

Figure 3. Western blot showing distinct profile of PrP^{res} in the 4 test-positive elk. Lanes: 1, positive control (wild caught elk from Colorado); 2, elk No. 2; 3, inoculum; 4, elk No. 4; 5, elk No. 1; and 6, elk No. 3. Samples were prepared as homogenates without (lanes 1, 4, 5 and 6) or after enrichment by PTA precipitation (lanes 2 and 3). Molecular weight markers (kDa) are shown on the left.

herd, and the prolonged incubation period or protection from disease may be mediated by additional or alternative genetic factors. Polymorphisms within and outside of the PRNP gene complex are under investigation.

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This study was carried out under the guidelines of the institutional Animal Care and Use Committee at the National Veterinary Services Laboratories. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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b. Seward Laboratory, Northampton, UK.
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