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## PRION PROTEIN GENE HETEROGENEITY IN FREE-RANGING WHITE-TAILED DEER WITHIN THE CHRONIC WASTING DISEASE AFFECTED REGION OF WISCONSIN

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**ABSTRACT:** Chronic wasting disease (CWD) was first identified in Wisconsin (USA) in white-tailed deer (*Odocoileus virginianus*) in February 2002. To determine if prion protein gene (*Prnp*) allelic variability was associated with CWD in white-tailed deer from Wisconsin, we sequenced *Prnp* from 26 CWD-positive and 100 CWD-negative deer. Sequence analysis of *Prnp* suggests that at least 86–96% of the white-tailed deer in this region have *Prnp* allelic combinations that will support CWD infection. Four *Prnp* alleles were identified in the deer population, one of which, resulting in a glutamine to histidine change at codon 95, has not been previously reported. The predominant allele in the population encodes for glutamine at codon 95, glycine at codon 96, and serine at codon 138 (QGS). Less abundant alleles encoded QSS, QGN, and HGS at the three variable positions. Comparison of CWD-positive with CWD-negative deer suggested a trend towards an over-representation of the QGS allele and an under-representation of the QSS allele.

**Key words:** Chronic wasting disease, prion protein, white-tailed deer, Wisconsin.

### INTRODUCTION

#### Chronic wasting disease

Chronic wasting disease (CWD) is the cervid form of an unconventional group of diseases known as transmissible spongiform encephalopathies (TSE). Prion diseases, or TSEs, are characterized by the accumulation of protease-resistant prion protein termed PrP<sup>TSE</sup> in the brains of affected animals. Protease-resistant prion protein accumulates via conversion of the normal cellular prion protein (PrP<sup>C</sup>) to the disease-associated isoform.

CWD was first detected in captive mule deer (*Odocoileus hemionus hemionus*) in Colorado (Williams and Young, 1980) and, subsequently, in free-ranging deer and elk (*Cervus elaphus*) in southeastern Wyoming and northeastern Colorado. In 2001, the range of the disease in wild deer extended to western Nebraska. As of February 2003, CWD had been identified in free-ranging deer in Illinois, New Mexico, Saskatchewan, South Dakota, Utah, and Wisconsin as well as Wyoming, Colorado, and Nebraska. It has also been identified in farm-raised elk and/or deer in Colorado, Nebraska, Alberta, Saskatchewan, Kansas,

Montana, Minnesota, South Dakota, Oklahoma, and Wisconsin.

In Wisconsin, CWD was first detected in free-ranging white-tailed deer (*Odocoileus virginianus*) in the south-central portion of the state in February 2002. In response, the Wisconsin Department of Natural Resources (DNR) initiated a surveillance program in the region. Of the initial 476 white-tailed deer tested using immunohistochemistry, 15 were brainstem (obex) and/or retropharyngeal lymph node positive for PrP<sup>CWD</sup>. By August 2002, 40/2,476 free-ranging deer had tested positive for CWD (J. Langenberg, pers. comm.).

#### Genetic susceptibility

The link between *Prnp* variability and susceptibility to prion diseases has been demonstrated in domestic animals, experimental animal models, and in human forms of the prion disease. A genetic susceptibility factor in mice, the scrapie incubation gene (*Sinc*), was identified by genetic and transmission studies (Dickinson et al., 1968). Subsequent molecular analyses identified a single amino acid change in the prion protein of mice exhibiting different incubation phenotypes (Westaway

et al., 1987). Similar to the *Sinc* gene in mice, the scrapie incubation period gene (*Sip*) was linked to scrapie susceptibility in sheep (Dickinson and Outram, 1988). Amino acid polymorphisms at positions 136, 154, and 171 in sheep PrP have been linked to development of both natural and experimental scrapie (Hunter et al., 1997).

Three mechanisms of contracting prion disease in humans are typically described: sporadic, familial, and iatrogenic. A fourth, the consumption of bovine spongiform encephalopathy (BSE) agent, appears to be the cause of variant CJD (vCJD). In all forms of human prion diseases, a *Prnp* polymorphism at codon 129 (encoding for methionine or valine) affects susceptibility or disease presentation (reviewed in Kovács et al., 2002 and Prusiner, 1999). In the case of vCJD, all confirmed cases have occurred in individuals homozygous for methionine at codon 129 (Asante et al., 2002). In addition to the codon 129 polymorphism, more than 20 different *Prnp* polymorphisms have been linked to familial forms of the disease in humans. It is unknown how these polymorphisms contribute to disease formation. Polymorphisms that result in PrP amino acid changes are strongly linked to unique phenotypic presentations of the disease.

#### PrP gene variability in cervids

Previous studies identified *Prnp* polymorphisms in elk and deer at codon positions 96, 132, 138, and 226 (O'Rourke et al., 1999; Raymond et al., 2000). Elk differ from white-tailed and mule deer at codon 226 and exhibit variability in codon 132. Both white-tailed and mule deer are polymorphic at codon 138, while heterogeneity at codon 96 is unique to white-tailed deer. Polymorphisms in *Prnp* could affect CWD susceptibility in cervids, impacting the degree to which cervid populations are vulnerable to disease transmission and progression. O'Rourke et al. (1999) detected a bias in Rocky Mountain elk homozygous for methionine at *Prnp* codon 132 among CWD-positive elk, with M/L heterozygous

and L/L homozygous elk being under-represented. Of 88 CWD-negative elk in Larimer County, Colorado, 60 (68%) were M/M; however, all 12 CWD-positive elk in the county were M/M. This study also found that 54% of the elk from a South Dakota facility were M/L. In contrast, only 26% of the CWD-positive elk were M/L, while 74% elk positive for CWD were M/M.

It is not known if *Prnp* polymorphisms affect the susceptibility of white-tailed deer to CWD. To address this question, we initiated a study to determine if specific *Prnp* polymorphisms are associated with the presence of CWD in the CWD-affected region of south-central Wisconsin. The prion protein gene was sequenced from 26 CWD-positive and 100 CWD-negative white-tailed deer from the south-central Wisconsin CWD Eradication Zone. Four *Prnp* alleles were identified. Specific *Prnp* alleles were observed to be associated with CWD-positive white-tailed deer.

## MATERIALS AND METHODS

### Tissues

All tissue samples were provided by the Wisconsin Department of Natural Resources and were obtained from deer harvested within the 1064 km<sup>2</sup> CWD-affected region in Dane and Iowa counties of south-central Wisconsin. Deer were harvested between March 2002 and September 2002 except two CWD-positive deer that were harvested in the fall of 2001. Blood and ear tissues were collected from deer heads previously sampled for CWD by the Wisconsin DNR. Obex and retropharyngeal lymph nodes were tested for the presence of PrP<sup>CWD</sup> using immunohistochemistry (IHC) at the National Veterinary Services Laboratories in Ames, Iowa (USA). Deer defined as CWD-positive stained positive for PrP<sup>CWD</sup> in the obex and/or retropharyngeal lymph nodes. Deer defined as CWD-negative were animals that were obex and lymph node IHC-negative for proteinase K resistant PrP (Julia Langenberg, pers. comm.).

### Isolation of genomic DNA

Genomic white-tailed deer DNA was isolated from blood using the DNA IQ System (Promega, Madison, Wisconsin). Manufacturer's instructions were followed with the exception of an additional lysis buffer wash. Genomic DNA

was isolated from ear tissue by incubating 10–25 mg tissue in 100  $\mu$ l digestion solution (800  $\mu$ l digestion buffer [10 mM EDTA, 0.1% SDS, and 1 mM Tris pH 9.0], 100  $\mu$ l 1M DTT, and 100  $\mu$ l proteinase K [18 mg/ml]) for 1–2 hr at 56 C, vortexing every 15 min. Samples were then centrifuged at 8,200 $\times$ G for 5 min. The resulting supernatant was added to 50  $\mu$ l DNA IQ lysis buffer and 7  $\mu$ l magnetic beads (Promega) and incubated for 5 min at room temperature. Samples were rinsed once with lysis buffer and three times with the wash buffer, using the magnetic stand between each wash to separate the beads from supernatant. Genomic DNA was then eluted from the beads as per the manufacturer's instructions.

#### Amplification and sequencing of the white-tailed deer *Prnp*

Primers for amplification of the entire open reading frame of white-tailed deer *Prnp* were designed based on the published sequence (GenBank accession # AF156185) using Lasergene<sup>®</sup> software. For amplification of the PrP coding region, primer CWD-21 (5'-ATAAGT-CATCATGGTGAAAAGCCAC-3' [forward]) and CWD-801 (5'-CTATCCTACTATGAGAAAATGAGGAAAAGA-3' [reverse]), or primers CWD-13 (5'-TTTTGCAGATAAGTCATCATGGTGAAA-3' [forward]) and CWD-LA (5'-AGAAGATAATGAAAACAGGAAGTTGC-3' [reverse]) were used. Polymerase chain reaction amplification conditions included initial denaturation of the sample at 95 C for 5 min, then amplification with PlatinumTaq (Invitrogen, Carlsbad, California, USA) for 10 cycles at 95 C (45 sec), 58 C (45 sec), and 72 C (1.5 min) followed by 25 cycles at 95 C (45 sec), 57 C (45 sec), and 72 C (1.5 min), and a final extension at 72 C (5 min). Amplified product was gel purified using QIAquick<sup>®</sup> kit (Qiagen, Valencia, California) and sequenced at the University of Wisconsin-Madison Biotechnology Center using dideoxy terminator sequencing. Primer pairs CWD-21, -801, -161 (5'-AGGGAAGTCCTGGAGGCAA CCGCTATCC-3' [forward]), and -418 (5'-CACCAAGGCCCTACTACTGCTCCA GC-3' [reverse]), or CDW-13, -LA, -161, and -418 were used for sequencing. Sequence was analyzed using Edview and MacVector 6.5 software.

#### Bae I digestion

The restriction endonuclease Bae I (New England Biolabs, Beverly, Massachusetts, USA) was used to confirm the presence of an A to C nucleotide (nt) change at nt 315. Amplification products (1  $\mu$ g) were incubated with five units Bae I (5' . . . 10/7 [N]ACNNNGTAPy C[N]

12/7 . . . 3') as per the manufacturer's instructions. Following digestion, Bae I was inactivated by incubating at 65 C for 20 min. Digestion products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light.

#### Statistical analysis

To estimate the proportion of the white-tailed deer population in the CWD-affected region that are genetically susceptible to CWD, we constructed a 95% confidence interval based on a large-sample approximation to the binomial distribution using the frequency of allelic combinations in the CWD-negative population.

## RESULTS

#### Prion protein variability in white-tailed deer

The PrP gene was sequenced from 26 CWD-positive and 100 CWD-negative free-ranging white-tailed deer from the 1064 km<sup>2</sup> CWD-affected region of Wisconsin. We identified 14 *Prnp* DNA polymorphisms. Three of these polymorphisms, at codon positions 95, 96, and 138, change the amino acid sequence of the prion protein. The majority of deer (87.3%) had at least one copy of the glutamine (95), glycine (96), and serine (138) PrP allele (QGS). This allele has been referred to as wild-type (Raymond et al., 2000). Other alleles identified in the 1064 km<sup>2</sup> affected region include QSS and QGN, both of which have been characterized elsewhere (Raymond et al., 2000). In addition, we identified a fourth, relatively rare allele, HGS, in three of the animals tested.

The adenine to cytosine change at nt 315 of the deer *Prnp*, resulting in a glutamine to histidine change at amino acid 95, produced a unique restriction endonuclease site, Bae I, on the genome. Amplified *Prnp* from deer heterozygous for the HGS allele exhibited appropriately sized digestion products (500 and 300 bp) (Fig. 1). Since both deer are heterozygous for histidine, one-half of the amplified product encodes a glutamine and did not digest with Bae I. Amplified *Prnp* from deer homozygous for glutamine at position

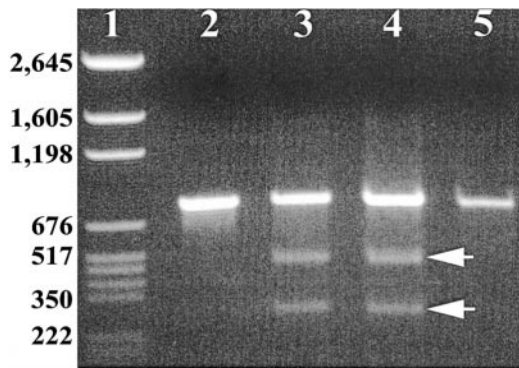


FIGURE 1. Bae I digestion of *Prnp* amplification products. Lane 1: molecular weight markers; lane 2: undigested amplification product from an HGS heterozygote; lanes 3 and 4: HGS heterozygous deer digested with Bae I; and lane 5: QGS homozygote digested with Bae I. Arrows indicate the Bae I digestion products.

95 was not cleaved by the restriction endonuclease.

All CWD-positive deer sequenced were QGS/QGS, QGS/QGN, QGS/QSS, or QSS/QGN allele combinations (Fig. 2). Sequence analysis of cloned amplification products from at least one of the putative double heterozygotes confirmed the assignment of the polymorphisms (data not shown). The combinations found in CWD-positive deer comprise 91% of the CWD-negative deer tested. When extrapolated at a 95% confidence interval,  $91 \pm 5\%$  of the CWD-negative deer in the affected region encode *Prnp* alleles that will facilitate infection.

The majority of PrP alleles in deer from CWD-positive (86.5%) and CWD-negative (68.0%) populations were QGS. Seventy-seven percent of the CWD-positive deer were QGS/QGS, whereas 47% of the CWD-negative deer were QGS/QGS. The QSS allele appears to be under-represented in the CWD-positive deer population. Twenty-four percent of the *Prnp* alleles are QSS in CWD-negative deer while comprising 9.6% of the PrP alleles in CWD-positive deer (Fig. 3). Deer homozygous for QSS represent 6% of the CWD-negative deer (Fig. 2). We have not

Allele Combinations	Infected	Uninfected
$\frac{QG}{QG} \frac{S}{S}$ QGS/QGS	20/26	47/100
$\frac{QG}{QS} \frac{S}{S}$ QGS/QSS	4/26	30/100
$\frac{QS}{QS} \frac{S}{S}$ QSS/QSS	0/26	6/100
$\frac{QG}{QG} \frac{S}{N}$ QGS/QGN	1/26	7/100
$\frac{QS}{QS} \frac{S}{N}$ QSS/QGN	1/26	7/100
$\frac{QG}{HG} \frac{S}{S}$ QGS/HGS	0/26	1/100
$\frac{QG}{HG} \frac{N}{S}$ QGN/HGS	0/26	1/100
$\frac{QS}{HG} \frac{S}{S}$ QSS/HGS	0/26	1/100

FIGURE 2. *Prnp* analysis of white-tailed deer from the 1064 km<sup>2</sup> eradication zone in Wisconsin. Four different alleles, representing four different PrP proteins, have been identified. The three amino acids listed with each allele represent the three variable positions at residues 95, 96, and 138.

identified a CWD-positive deer with the QSS/QSS allele.

## DISCUSSION

This study indicates that the majority of white-tailed deer in the CWD-affected area of south-central Wisconsin have *Prnp* allelic combinations that are found in CWD-positive deer suggesting that there is little to no molecular barrier to CWD infection in this region. The *Prnp* allelic

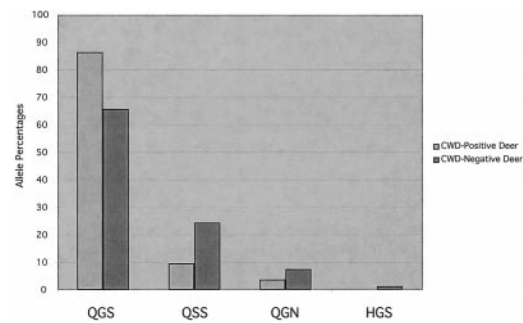


FIGURE 3. Allelic frequency of the four *Prnp* alleles identified in white-tailed deer from Wisconsin. The *Prnp* coding region was sequenced from 26 CWD-positive (52 alleles) and 100 CWD-negative (200 alleles) deer. All deer were obtained from the 1064 km<sup>2</sup> CWD eradication zone in south-central Wisconsin.

combinations present in the CWD-positive deer suggest that at least 86% to 96% of the white-tailed population in the affected region are susceptible to CWD.

#### Susceptibility factors

The QGS and QSS alleles constitute the majority of the PrP alleles (92.8%) in deer from the eradication zone that were tested. Although no significant differences were found, trends suggest that QSS is under-represented in the CWD-positive population. We found this disparity of allelic representation somewhat surprising given that previous *in vitro* cell-free conversion data indicated little or no molecular barrier to conversion of the QSS allele to the proteinase K resistant form (Raymond et al., 2000). It is possible that the presence of the QSS allele slows disease progression by reducing the number of deer harboring detectable levels of infectious agent. We have, however, identified one CWD-positive deer that was QSS/QGN, indicating that either the QSS and/or the QGN proteins can be converted to the infectious form.

#### Potential for CWD strains

Deer homozygous for the QGS allele comprised the majority of the CWD-positive animals sequenced. White-tailed deer encoding QGS/QGN and QGS/QSS PrP variants have also tested positive for CWD. It is possible, in heterozygous deer, that the QSS and/or QGN proteins are being converted to an infectious form. Identification of a CWD-positive QSS/QGN deer suggests there is infectious PrP agent present in the population containing either the QSS or QGN protein. In addition to potentially changing the susceptibility/resistance of deer that carry these alleles, variations in the primary protein sequence may also affect transmission properties of the agent. For example, an animal of another species, resistant to infectious agent passed in QGS/QGS deer, may be susceptible to agent from a homozygous or heterozygous deer carrying another allele.

It is not known whether different CWD strains exist. The inability to efficiently transmit the disease to rodents has limited such studies. Cell-free conversion experiments in which polymorphic deer PrP<sup>C</sup> proteins were incubated in the presence of PrP<sup>CWD</sup> suggest that distinct differences in conversion efficiencies exist (Raymond et al., 2000). In sheep, a single amino acid change in PrP<sup>Sc</sup> resulted in altered abilities to convert PrP<sup>C</sup> by cell-free conversion (Bossers et al., 2000).

#### Management implications of this study

The geographic range of CWD is expanding. The apparent resistance of sheep, with specific *Prnp* alleles, to specific strains of scrapie has led to suggestions that genetic resistance may be a means of managing CWD in free-ranging white-tailed deer. Our data, as well as data from elk *Prnp* analyses, suggest that genetic resistance, based on changes in the prion protein, is not a viable option as a management tool.

#### CONCLUSION

A number of factors contribute to the epidemiology of CWD in free-ranging white-tailed deer including: 1) contagious nature of the CWD agent, 2) extended preclinical stage of the disease, 3) deer density and movement, 4) resistance of the infectious agent to inactivation, 5) degree of human intervention (both positive and negative) and 6) genetic susceptibility to the disease. Given that the majority of the deer present in the CWD-affected region of south-central Wisconsin are susceptible to CWD and the contagious nature of the disease, a continued increase in the prevalence and the range of CWD are likely.

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