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Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer

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Chronic wasting disease (CWD) status and PrP genotypes were determined for a group of 133 wild white-tailed deer in a 780 acre enclosure in western Nebraska, USA. Approximately half of the deer tested showed evidence of PrP^d in the brainstem or lymphoid tissues. Four *PRNP* alleles encoding amino acid substitutions were identified, with substitutions at residues 95 (Q→H), 96 (G→S) or 116 (A→G), each with serine (S) at residue 138. In addition, a processed pseudogene with two alleles encoding five or six copies of the octapeptide repeat was identified in 26 % of the deer. Both alleles encoded asparagine (N) at residue 138. The functional gene alleles sorted into five major diploid genotypes and four rare genotypes. Although all five major diploid genotypes were found in deer with CWD, unaffected deer were less likely to have the allele QGAS and more likely to have QSAS compared with CWD-affected deer. Late-stage disease (PrP^d in brainstem) was noted in deer less than 1 year of age, although no single genotype was associated with this rapid neuroinvasion. Early-stage disease (PrP^d distribution limited to the lymphoid system) was observed in deer estimated to be more than 5 years old, suggesting that they were infected as adults or that the incubation time might be extremely long in some individuals. The pseudogene was found in deer of all major *PRNP* genotypes and was not correlated with CWD status. The large number of susceptible genotypes and the possibility of adult-to-adult transmission suggest that much of the white-tailed deer population may be at risk for disease following exposure to CWD, despite the association of specific genotypes with CWD noted here.

INTRODUCTION

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) of North American cervid ruminants. CWD is reported at low incidence in farm-raised and free-ranging white-tailed deer (*Odocoileus virginianus*), mule deer (*O. hemionus*) (Miller *et al.*, 2000) and Rocky Mountain elk (*Cervus elaphus*) (Spraker *et al.*, 1997; Williams & Young, 1980) in several US states and Canadian provinces. Other naturally occurring North American TSEs include geographically widespread scrapie of sheep (Wineland *et al.*, 1998), very low numbers of scrapie-infected goats (Valdez *et al.*, 2003) and rare epidemic outbreaks of transmissible mink encephalopathy in farm-raised mink (Hartsough & Burger, 1965).

The TSEs are a diverse family of fatal neurodegenerative diseases characterized by accumulation of PrP^d, an abnormal disease-specific isoform (Prusiner, 1982) of the normal host protein PrP^c (Basler *et al.*, 1986). Transmission and pathogenesis of the TSEs appear to involve a novel mechanism in which PrP^d, a major protein component in tissue extracts from infected animals, catalyses the conversion of PrP^c to the pathogenic PrP^d molecule through a series of aggregation and post-translational secondary changes (Caughey *et al.*, 1990; Horiuchi & Caughey, 1999; Safar *et al.*, 1994). The 'protein only' model is supported by experiments demonstrating that expression of PrP^c in the host is a necessary prerequisite for disease (Bueler *et al.*, 1993) and that the primary amino acid sequence of PrP^c is associated with relative susceptibility to natural TSE infection in some species. Residues 136, 154 and 171, alone or in combination, control relative susceptibility and incubation

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time in sheep (Belt *et al.*, 1995; Bossers *et al.*, 1996; Clouscard *et al.*, 1995; Goldmann *et al.*, 1994a, b; Hunter *et al.*, 1992, 1994; O'Rourke *et al.*, 1997; Westaway *et al.*, 1994). Predisposing genotypes in elk with CWD (O'Rourke *et al.*, 1999) and in humans with iatrogenic, sporadic and variant Creutzfeldt–Jakob disease (Collinge *et al.*, 1991, 1996; Palmer *et al.*, 1991) are associated with mutations at a single site (elk codon 132 and the corresponding human codon 129). Genetic analysis of susceptible or predisposing prion protein precursor gene (*PRNP*) alleles in mule deer is complicated by the nearly universal presence of an unexpressed processed pseudogene (*PRNPψ*) in which exon 3 encodes a polymorphic open reading frame (ORF) (Brayton *et al.*, 2004). In this study, we examined the *PRNP* functional gene and pseudogene alleles in a herd of white-tailed deer with a high prevalence of naturally occurring CWD to investigate the possibility of predisposing genotypes in this species.

METHODS

Animals. Tissues were collected from white-tailed deer on a ranch in Sioux county in the northwest corner of Nebraska, USA, within the CWD endemic area. In 1992, an area of pine-covered steep hills containing many deep ravines was enclosed with a 3 m net and barbed-wire fence. An estimated 20–30 free-ranging white-tailed deer and a smaller number of mule deer were inadvertently fenced in this enclosure. This herd was allowed to multiply into a population as high as 300 head over the next 10 years. Food plots, creep feeders with trace minerals and hay were used to supplement the deer in this enclosure. Some losses to enzootic haemorrhagic disease of deer in the enclosure coincided with similar losses in deer outside the fence. No deer in poor body condition or with symptoms suggestive of CWD were noted. From 1992 to 2001, animals were removed during guided hunts that met all of the State of Nebraska Game and Parks Division season and permit requirements. A group of bull elk was placed in the deer enclosure in 1998 and those not harvested by hunters were moved to an adjacent pasture. In 2001, CWD surveillance was initiated and positive deer were found in the first group sampled. The entire enclosure and several acres at the perimeter were depopulated in early 2002 under an agreement between the property owner and the Nebraska Game and Parks Commission. All deer ($n=172$) were removed from within the fenced area. Thirty-nine animals were excluded from the study because the submission lacked either a complete tissue set (formalin-fixed brain, tonsil and retropharyngeal lymph node) for CWD status determination or a frozen tissue sample suitable for genetic analysis. Diagnostic testing was performed by the Colorado State Veterinary Diagnostic Laboratory or the Wyoming Veterinary Diagnostic Laboratory. All animals were in good body condition when killed. Most of the deer were killed in late winter (January–March) 2002. White-tailed deer breed seasonally and fawns were 7–10 months of age when the herd was depopulated. Age was estimated by tooth eruption in 36 of the 88 adult animals; the remaining 52 were recorded as 'adult'.

PCR and DNA sequence analysis. DNA was extracted from frozen brain tissue using a commercial kit (Q-Biogene) following the manufacturer's instructions. Primer pairs specific for amplification of *PRNP* and *PRNPψ* were derived from the corresponding sequences from mule deer (GenBank accession nos AY330343 and AY371694). Intron–exon borders were derived from the sequences of bovine *PRNP* cDNA and genomic DNA (GenBank accession nos AB001468 and AJ298878) and cDNA from mule deer (K. I. O'Rourke, unpublished data). PCR amplification was performed using a universal prion primer pair amplifying an 800 bp fragment including the entire ORF in exon 3 of *PRNP* and *PRNPψ* (forward primer 247 5′-atggtgaaaagccacataggcag-3′, beginning with the initiation site for PrP translation and reverse primer 224 5′-agaagataatgaaaacaggaag-3′, beginning 8 bp after the termination codon); a primer pair specific for the functional gene (forward primer 223 5′-acaccctcttattttgcag-3′ in intron 2 and reverse primer 224), yielding an 830 bp product; and two primer pairs specific for the pseudogene (forward primer 379 5′-aagaaaattcctgagagagcat-3′ containing the pseudogene flanking repeat sequence and the adjacent 8 bp from exon 1 or forward primer 369 5′-caaccaagtcgaagcatct-3′ from exon 2; reverse primer 224 was used in both cases). Amplification of DNA from deer with *PRNPψ* yielded a product of 950–974 bp with primers 379/224 and 875–899 bp with primers 369/224, depending on the octapeptide repeat number. Although primers 369/224 annealed to *PRNP* and to *PRNPψ*, the intervening intron in *PRNP* (deleted in *PRNPψ*) precluded efficient amplification of the functional gene and therefore the primer pair selectively amplified *PRNPψ* under the conditions used. All PCR reactions were as follows: 95 °C for 5 min, followed by 30 cycles of denaturation (95 °C, 30 s), annealing (54 °C, 30 s) and extension (72 °C, 59 s) followed by an extension cycle (72 °C, 7 min) under standard buffer conditions with 2·5 mM $MgCl_2$ (Qiagen). PCR products were analysed on 1 % agarose ethidium-bromide-stained gels. *PRNPψ* amplification reactions were scored for the presence or absence of a pseudogene-specific band.

PCR products were purified by Exo/SAP (USB Corporation) to remove unincorporated dNTPs and primers, then sequenced on an ABI Prism 377 DNA Sequencer with Big Dye Terminator chemistry (PE-Applied Biosystems) using forward primer 245 (5′-gccaaccgctatccacctca-3′) and reverse primer 12 (5′-ggtggtgactgtgtgttgcttga-3′) (Amplicon Express). PCR products were cloned for sequencing of each *PRNP* allele and all *PRNPψ* products using a TOPO TA cloning system (Invitrogen) according to the manufacturer's instructions and sequenced with primers T3 and T7 (MWG Biotech). In this study, *PRNP* alleles were identified by the deduced amino acids at positions 95 (glutamine, Q, or histidine, H), 96 (glycine, G, or serine, S), 116 (alanine, A, or glycine, G) and 138 (serine, S, or asparagine, N), sites of the three coding changes in the functional gene, and the *PRNP*/*PRNPψ* marker at codon 138 of *Odocoileus* spp. (S in *PRNP* and N in *PRNPψ*). *PRNPψ* alleles were identified by the number of octa/nonapeptide repeat units (five or six).

CWD diagnosis. CWD was diagnosed by monoclonal antibody immunohistochemistry as described previously (Spraker *et al.*, 2002a). Deer were considered CWD-positive if PrP^d was detected in the dorsal motor nucleus of the vagus nerve (DMNV), the tonsil or the retropharyngeal lymph node. Animals were considered to be in early disease if the tonsil or retropharyngeal node was positive but no immunostaining was evident in the DMNV (Spraker *et al.*, 2002b). Animals were considered to be in late disease if PrP^d was detected in brain as well as lymphoid tissue. Deer with no detectable PrP^d in brain or nodes were considered 'No PrP^d'. These animals were not considered CWD-free because of the long undefined incubation period between infection and accumulation of detectable PrP^d.

Statistical analysis. Frequencies of CWD associated with specific *PRNP* alleles were assessed by χ^2 analysis of contingency tables, using Yates continuity correction where appropriate (for comparisons with a single degree of freedom). Confidence intervals were calculated for odds ratios of CWD in the major genotypes detected in the study population, using the presumed wild-type genotype QGAS QGAS as the reference genotype (Armitage & Berry, 1994).

RESULTS

PRNP alleles

The ORF of *PRNP* was determined by DNA sequence analysis of PCR products generated using a primer pair specific for the functional gene. Alleles were confirmed by sequence analysis of cloned amplicons. The previously reported polymorphisms at codons 95 (caa/cat, Q→H), 96 (ggt/agt, G→S) (Raymond *et al.*, 2000; Johnson *et al.*, 2003) and 116 (gca/gga, A→G) (Heaton *et al.*, 2003) were noted. Silent changes (cgc/cgt at codon 51, aac/aat at codon 146 or ggt/gga at codon 81) were identified in a small number of animals. The frequencies of the functional gene alleles QGAS, QSAS, QGGS and HGAS are shown in Table 1. The most common alleles, QGAS and QSAS, represented nearly 90 % of the total (frequencies 0·50 and 0·36, respectively). Alleles QGGS and HGAS were rare (frequencies 0·13 and 0·011). All functional gene alleles encoded five octa/nonapeptide repeat units with nonapeptides P(Q/H)GGGGWGQ flanking three octapeptides (PHGGGWGQ).

PRNPψ alleles

The cervid prion pseudogene, *PRNPψ*, bears the hallmarks of a processed pseudogene, lacking introns and flanked by direct repeats (Mighell *et al.*, 2000). The white-tailed deer *PRNPψ* was amplified using each of two pseudogene-specific primer pairs: forward primer 379 incorporating the 5′ pseudogene repeat (Fig. 1) and the 5′ end of the truncated exon 1, or forward primer 369 from exon 2, each paired with the 3′ universal prion reverse primer at the end of the ORF. Products were evaluated by agarose gel electrophoresis (Fig. 2) and all amplicons sequenced directly or after TOPO cloning. Of 133 samples, 35 (26 %) yielded an amplification product with the pseudogene-specific primers. Sequence analysis of TOPO-cloned products demonstrated that the organization of *PRNPψ* in white-tailed deer (GenBank accession no. AY425673) was similar to that in mule deer (GenBank accession no. AY371694), containing a truncated exon 1 contiguous with exon 2 and exon 3 sequences (Fig. 1). The 5′ sequence flanking the pseudogene was identical to that of mule deer [5′-(aa)gaaaattcctgaga-3′]. This sequence was identified at the 5′ end of the white-tailed deer pseudogene. Because of the similarity with the mule deer pseudogene, it is very likely that this flanking sequence is a part of a repeat also

Table 1. PrP functional and pseudogene allelic frequencies of 133 white-tailed deer in a herd with a high prevalence of CWD

Allele	No. of homozygous deer	No. of heterozygous deer*	Allelic frequency†
Functional alleles			
QGAS	26	80	0·50
QSAS	12	71	0·36
QGGS	2	32	0·13
HGAS	0	3	0·011
Pseudogene alleles			
None	98	0	0·74
Five repeats	29	0	0·22
Six repeats	6	0	0·04

*Heterozygous deer are shown twice, once for each allele.
†Allelic frequency = [(homozygous deer × 2 alleles/deer) + (heterozygous deer × 1 allele/deer)]/(total deer × 2 alleles/deer).

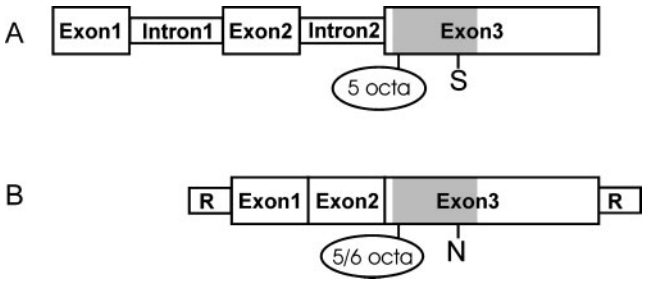

Fig. 1. Organization of the cervid functional *PRNP* gene and the cervid prion pseudogene *PRNPψ* (not drawn to scale). (A) The functional gene includes three exons with intervening introns. The entire ORF (shown in grey) is found in exon 3 and encodes five octa/nonapeptide repeat units and serine (S) at codon 138 in all alleles. (B) The cervid pseudogene contains the classic hallmarks of a processed retrotransposon, lacking introns and flanked by a direct repeat (R; the 3′ repeat is inferred from similarity to the mule deer pseudogene). The pseudogene encodes either five octa/nonapeptide repeat units identical to those in the functional gene or six repeat units, with an additional 24 bp repeat unit inserted after the second unit. Both *PRNPψ* alleles encode asparagine (N) at codon 138.

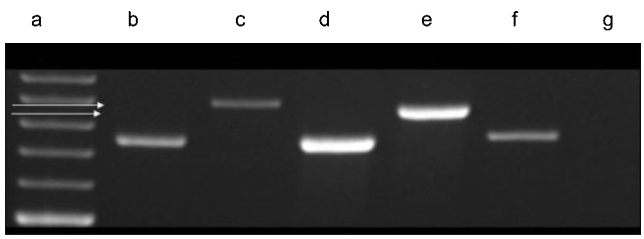

Fig. 2. Amplification of genomic DNA with primers specific for the functional gene *PRNP* (lanes b, d and f, primers 223/224) and the cervid pseudogene *PRNPψ* (lanes c, e and g, primers 379/224) from deer 115-261 (lanes b and c) with a pseudogene encoding six octapeptide repeats (upper arrow), deer 115-1 (lanes d and e) with a pseudogene encoding five octapeptide repeats (lower arrow) and deer 115-84 (lanes f and g) with no pseudogene. Molecular mass markers (lane a) are 1100, 1000, 900, 800, 700 and 600 bp.

found at the 3′ terminus of the pseudogene. The *PRNPψ* sequence in white-tailed deer was identical to that of the most common allele in mule deer and lacked the heterogeneity of the mule deer *PRNPψ* (Brayton *et al.*, 2004). No single base pair changes were identified among the white-tailed deer *PRNPψ* sequences. Variation was observed in the octapeptide repeat number in the pseudogene, with either five ($n=29$ deer) or six ($n=6$ deer) repeat units observed. The longer allele had a 24 bp insertion after the second repeat unit; the other five repeat units were identical to those in the shorter pseudogene allele and in the functional gene. All functional gene alleles encoded serine (S) at codon 138 and all pseudogene alleles encoded asparagine (N), as observed in mule deer.

CWD in adult deer and fawns

A hundred and thirty-three white-tailed deer in the study were killed after CWD was diagnosed in the deer within the fenced area. Paired samples of formalin-fixed tissue for CWD diagnosis and frozen tissue for DNA sequence analysis were collected. Fifty per cent (67/133) of deer were diagnosed with CWD (Table 2) using an immunohistochemical assay for PrP^d in formalin-fixed, paraffin-embedded brain and lymphoid tissues. Five of the CWD-positive deer were fawns, less than 1 year of age. Early CWD (PrP^d detected in the tonsil or retropharyngeal node but not brain) was diagnosed in 14 deer (12 adults ranging from 1·5 to more than 5 years of age and two fawns). Late CWD (PrP^d detectable in brain as well as lymphoid tissues) was diagnosed in 53 deer (50 adults ranging in age from 1·5 to 7 years of age and three fawns). None of the CWD-positive deer showed clinical signs of the disease (weight loss, hypersalivation, disorientation) or gross changes consistent with CWD (serous atrophy of fat) at necropsy.

Diploid genotypes of deer of varying age and disease status

Diploid genotypes and CWD status for the 133 deer in this study are shown in Table 2. Nine of the 10 potential diploid genotypes were identified in the herd, lacking only the deer homozygous for the rare HGAS allele. CWD was diagnosed in deer with the three most commonly occurring genotypes (QGAS QGAS, QGAS QSAS and QGAS QGGS) and three of the less common genotypes (QSAS QGGS, QSAS QSAS, QGGS HGAS). Late-stage disease was seen in fawns and adults with genotypes QGAS QSAS and QGAS QGGS. Adult deer with early disease were identified in three genotypes (QGAS QGAS, QGAS QSAS and QSAS QGGS). Deer homozygous for the two major alleles (QGAS and QSAS) were found in the CWD population, although only one fawn and one adult homozygous for QSAS were identified.

The frequencies of CWD differed among deer with the five major diploid genotypes (i.e. the first five rows of Table 2) ($\chi^2=12{\cdot}2$, 4 df, $P=0{\cdot}016$). The other genotypes were too rare for meaningful analysis. The strongest association

Table 2. *PRNP* and disease status of white-tailed deer with early or late CWD

A hundred and thirty-three white-tailed deer were evaluated for diploid functional *PRNP* genotype, the presence of the cervid prion pseudogene *PRNPψ* and CWD status.

PRNP genotype	No. in herd (%)*	No. with CWD (%)†	Odds ratio (95 % CI)	CWD status (fawns, adults)		
				Early‡	Late§	No PrP^d‖
QGAS QGAS	26 (20)	18 (27)	Reference genotype	3 (1, 2)	15 (0, 15)	8 (5, 3)
QGAS QSAS	59 (44)	33 (56)	0·564 (0·212–1·501)	6 (0, 6)	27 (2, 25)	26 (19, 7)
QGAS QGGS	20 (15)	10 (15)	0·444 (0·133–1·489)	0	10 (1, 9)	10 (6, 4)
QSAS QGGS	11 (8)	3 (4)	0·167 (0·035–0·789)	3 (0, 3)	0	8 (4, 4)
QSAS QSAS	12 (9)	2 (3)	0·089 (0·016–0·502)	1 (1, 0)	1 (0, 1)	10 (4, 6)
QGGS QGGS	2 (1·5)	0	ND	0	0	2 (2, 0)
QGGS HGAS	1 (1)	1 (1)	ND	1 (0, 1)	0	0
HGAS QGAS, HGAS QSAS	2 (1·5)	0	ND	0	0	2 (2, 0)
PRNPψ						
No *PRNPψ*	98 (74)	50 (51)		11 (1, 10)	39 (3, 36)	48 (19, 29)
PRNPψ	35 (26)	17 (49)		3 (1, 2)	14 (0, 14)	18 (12, 6)

*Number and percentage of deer tested ($n=133$) of each diploid functional (*PRNP*) and pseudogene (*PRNPψ*) genotype.
†Number and percentage of CWD-positive deer ($n=66$) of each diploid functional (*PRNP*) and pseudogene (*PRNPψ*) genotype.
‡Early CWD defined as PrP^d immunostaining in lymphoid tissues but not brain.
§Late CWD defined as PrP^d immunostaining in lymphoid tissues and brain.
‖No PrP^d defined as no PrP^d immunostaining in either lymphoid tissue or brain.
ND, Not determined.

detected was between CWD occurrence and carriage of the QGAS allele ($\chi^2=11{\cdot}3$, 1 df, $P<0{\cdot}001$). Deer either haploid ($\chi^2=6{\cdot}52$, 1 df, $P=0{\cdot}01$) or diploid ($\chi^2=9{\cdot}36$, 1 df, $P<0{\cdot}01$) for the QGAS allele were more frequently affected by CWD compared with deer lacking this allele. Correspondingly, a negative association was observed between CWD and carriage of one or two QSAS alleles ($\chi^2=6{\cdot}98$, 2 df, $P<0{\cdot}05$). No association was detected between carriage of a *PRNP* pseudogene allele and CWD ($\chi^2=0{\cdot}003$, $P>0{\cdot}05$). Sample sizes were too small for analysis of all genotypes. Odds ratios and 95 % confidence intervals (CI) for the occurrence of CWD in the most frequently occurring diploid genotypes, using the presumed wild-type allele QGAS homozygous deer as the reference group, are shown in Table 2. In spite of the small sample size, the 95 % CI showed a decrease in the odds ratio for deer with the QSAS allele and lacking the QGAS allele when compared with deer with the presumed wild-type QGAS homozygous genotype. Homozygous QSAS deer had a lower odds ratio (0·089, 95 % CI 0·016–0·502) than did heterozygous QSAS QGGS deer (0·167, CI 0·035–0·789). The presence of the QGAS allele resulted in similar odds ratios for heterozygous QSAS (0·564, 95 % CI 0·212–1·501) or QGGS (0·444, 95 % CI 0·133–1·489) deer.

DISCUSSION

Relative genetic susceptibility to the TSEs is based on the supposition that variation at critical residues in PrP^c increases the efficiency of conversion to the PrP^d isoform. The post-translational change from PrP^c to PrP^d is thought to be a two-step process of PrP^c/PrP^d aggregation (Horiuchi & Caughey, 1999) followed by conversion of PrP^c to the β-sheet-enriched protease-resistant protein (Cohen & Prusiner, 1998). Amino acid substitutions that contribute to the efficiency of the aggregation step or the stability of the disease-associated isoform (Supattapone *et al.*, 2001) are expected to be associated with increased susceptibility or decreased incubation time. In ruminant herbivores with naturally occurring TSEs, susceptibility to natural challenge is estimated from the proportion of affected and unaffected animals of each genotype in populations with a presumed uniform exposure to the disease-causing agent. The low prevalence of CWD in free-ranging populations of deer and elk complicate a similar analysis of genetic susceptibility to CWD. We identified a group of wild deer, confined to a single large area, with CWD diagnosed in approximately 50 % of the animals. Examination of the relationship between CWD and *PRNP* genotype under these conditions of heavy natural challenge was performed to identify susceptible and predisposing genotypes.

The ORF of the *PRNP* gene was more variable in this group of deer than was reported previously for free-ranging elk (O'Rourke *et al.*, 1999) or mule deer (Brayton *et al.*, 2004) and was duplicated in the cervid prion pseudogene *PRNPψ* as described previously in mule deer (Brayton *et al.*, 2004). The presumed wild-type allele (QGAS) and three alleles with coding changes at codons 95 (H), 96 (S) or 116 (G) were identified in the functional gene. In addition, a coding change at codon 138 distinguished *PRNP* (138S) from *PRNPψ* (138N) in this sample set. Both the location and potential charge shift of the three amino acid replacements in the functional gene are of interest. The H/Q polymorphism at position 95 results in substitution of a neutral for a polar residue but is commonly reported in the deduced amino acid sequences from the *PRNP* gene among and within a large number of species (Schätzl *et al.*, 1995; Van Rheede *et al.*, 2003; Wopfner *et al.*, 1999). Residue 96, encoding G or S, is adjacent to the final octapeptide repeat in a region poorly conserved among the mammalian species examined, with insertions and single base changes reported (Van Rheede *et al.*, 2003). Although there was no evidence for resistance to disease in deer with any of the major genotypes in this study, deer lacking the QGAS allele and carrying one or two copies of the allele encoding 96S (QSAS) were less likely to be found in the CWD population than deer with the QGAS allele on one or both chromosomes. This finding is consistent with data from deer in Wisconsin (Johnson *et al.*, 2003), although in that study universal prion primers were used and the functional genotype could not be determined for some animals from the data shown. The sample sizes in both studies are relatively small (26 in Wisconsin, 67 in this study). If larger sample sizes support these observations, however, the pattern of genetic susceptibility in white-tailed deer would be similar to that observed in Rocky Mountain elk, in which a predisposing genotype is reported (O'Rourke *et al.*, 1999), although all major genotypes are susceptible to CWD (Spraker *et al.*, 2004). In contrast, *PRNP* polymorphisms in sheep result in the lack of disease in virtually all sheep with resistant genotypes (Belt *et al.*, 1995; Bossers *et al.*, 1996; Clouscard *et al.*, 1995; Goldmann *et al.*, 1994a, b; Hunter *et al.*, 1992, 1994, 1997; O'Rourke *et al.*, 1997; Westaway *et al.*, 1994).

The polymorphism at codon 116 of the functional gene is of interest. The alanine (A) residue at this position is conserved across a large number of eutherian orders (Van Rheede *et al.*, 2003) and previously reported to be variant only in the tenrec, an insectivorous mammal found only on the island of Madagascar. Residue 116 (human 113) is in a potential membrane-spanning domain (Hegde *et al.*, 1998) of PrP^c, immediately adjacent to a proposed cleavage site for cytosolically derived protease but protected from proteolysis by its insertion through the membrane of the endoplasmic reticulum (Hegde *et al.*, 1998). The A→G mutation at residue 116 in white-tailed deer results in replacement of a hydrophobic, aliphatic residue with a polar residue and may affect the intracellular processing and transport of the PrP precursor. However, in this study, the 116G allele was found in 13 CWD-positive heterozygous deer, demonstrating that the allele does not provide protection under these conditions. Two 116G homozygous animals were identified (one adult and one fawn), arguing against an early lethal effect of the mutation. Neither

homozygous deer had detectable PrP^d or neurodegenerative changes.

The cervid prion pseudogene in white-tailed deer in this study appeared at low frequency in comparison with the pseudogene in *O. hemionus*, the mule deer (Brayton *et al.*, 2004) and black-tailed deer (subspecies of *O. hemionus*) (unpublished data). *PRNPψ* was characterized by a polymorphism not detected in the functional gene and heterogeneity was limited to a variable octapeptide repeat number. The low frequency and lack of heterogeneity of the white-tailed deer pseudogene could be due to inbreeding within this confined herd, to polymorphisms in the primer binding sites resulting in detection of a limited subset of alleles or to a relatively recent introduction of the pseudogene into the genome of white-tailed deer. Although the founder group in the study herd was estimated at fewer than 20 animals, loss of heterogeneity in the pseudogene due to inbreeding is an unlikely explanation because all functional alleles identified in the Wisconsin (Johnson *et al.*, 2003), Mississippi (unpublished data) and Wyoming (Heaton *et al.*, 2003) groups are represented in this herd. Furthermore, using the 138N polymorphism as a marker for *PNRPψ* in the Wisconsin and Wyoming studies, pseudogene frequencies are similar among the groups. Polymorphisms in the PCR primer-binding sites cannot be ruled out. However, the pseudogene was detected with two primer pairs in all deer in which the 138N mutation was detected with the universal prion primer pair, suggesting that failure to amplify a novel *PRNPψ* allele is not likely. The third explanation is the appearance of the pseudogene in *O. hemionus* (mule deer and other black-tailed deer) after divergence from white-tailed deer, with limited and relatively recent introgression into the white-tailed deer genome through hybridization of ancestral sympatric populations. Although the evolution and taxonomy of the dozens of subspecies of white-tailed and black-tailed deer in the Americas are complex, examination of mitochondrial DNA and Y-linked genes supports the model that white-tailed deer and black-tailed deer diverged on the North American continent during the Pleistocene, with subsequent hybridization of sympatric populations resulting in appearance of mule deer (reviewed by Geist & Francis, 1999). Subsequent hybridization between mule deer and white-tailed deer probably occurred (and continues to occur) infrequently, with a low level of introgressive hybridization of maternal DNA from mule deer and black-tailed deer into white-tailed deer populations within limited geographic areas (Cronin, 1991).

The *PRNPψ* alleles in this study and the previous study of mule deer (Brayton *et al.*, 2004) invariably encoded 138N and an expansion of the octapeptide repeat number was observed in some deer. These changes were not seen in the functional gene, leaving unresolved the question of whether the changes occurred in the ancestral functional gene before the duplication event or in the processed pseudogene after duplication. The substitution of N for S in the prion protein is common within and among species and functional alleles encoding 138N are common in fallow deer (*Dama dama*) and reindeer (*Rangifer tarandus*) (unpublished data). Likewise, a variable octapeptide repeat number is common in some breeds of cattle. There are several dozen subspecies of *Odocoileus* in the Americas and genetic examination of additional populations should demonstrate whether the changes noted in the pseudogene in this study are characteristic of the processed pseudogene or are found in functional alleles as well. No evidence of the pseudogene was found in small populations of New World moose (*Alces alces*), holoarctic reindeer, Old World Rocky Mountain elk (*C. elaphus*) or captive Asian fallow deer (unpublished data). If *PRNPψ* arose after evolutionary radiation of *Odocoileus* species in the New World, this pseudogene is not likely to be found in more distantly related members of the order Artiodactyla such as sheep, cattle or goats. Novel pseudogenes may yet be found in some populations of domestic livestock through judicious selection of primer pairs optimized for pseudogene discovery. However, processed pseudogenes are not typically transcribed or translated (Mighell *et al.*, 2000) and are therefore not expected to affect susceptibility to a protein-based disorder.

All major *PRNP* diploid genotypes were represented in the CWD group in this study. We addressed the possibility of genetically controlled differences in incubation time (the period from infection until accumulation of detectable PrP^d in the brain) by examining fawns (animals under 1 year of age) for late disease (PrP^d in the brain). Using this crude measure, we identified three fawns (QGAS QSAS and QGAS QGGS genotypes) with late disease, demonstrating an extremely rapid course in those animals. PrP^d is thought to accumulate first in the lymphoid tissues of the alimentary tract (Sigurdson *et al.*, 1999), with subsequent neuroinvasion and transport through the splenic or vagus nerves to the brain. In experimental rodent models, the incubation time and neuroinvasion rate are associated with a number of variables, including *PRNP* polymorphisms (Dickinson & Outram, 1973), innervation and PrP^c production in lymphoid tissues (Mabbott *et al.*, 2000; Glatzel *et al.*, 2001), and route of infection (Bartz *et al.*, 2003). Transgenic mice with cervid *PRNP* genes may be an appropriate laboratory model for examining the extremely rapid neuroinvasion seen in some genotypes.

CWD in mule deer is typically associated with weight loss, ataxia, hypersalivation, polyuria and polydipsia (Williams & Miller, 2002) and similar signs have been reported in captive white-tailed deer held in research settings (M. W. Miller, personal communication). In spite of an infection rate of nearly 50 % in this herd, no clinical evidence of CWD was noted in live deer by the property owner or hunters or at necropsy. Losses to intercurrent viral diseases were extensive during some years and the terrain provided adequate cover for sick animals to evade detection prior to death, possibly masking the presence of a TSE in this herd.

Alternately, the clinical course in these white-tailed deer may have been more acute than described in mule deer due to differences in the biology of the cervid host, the strain of TSE agent in this herd or the dose of the disease-causing agent in this confined facility. Additional observations on clinical CWD in white-tailed deer held in captive settings will be necessary to address the spectrum of phenotypes of TSE infection in cervids.

PRNP alleles and diploid genotypes of white-tailed deer with CWD identify those associated with CWD susceptibility. In contrast, disease resistance is considerably more challenging to define in free-ranging populations for several reasons. First, equivalent exposure to the disease-causing agent cannot be demonstrated across the population. Although the deer in this study were removed from a single fenced area, it is likely that the herd spent part of the year in small maternally related groups, congregating at feed plots and at natural watering areas. CWD transmission routes are not known and the relative efficiency of transmission through deer-to-deer contact or through shared feed and water sources cannot be determined. Microsatellite analysis of DNA from the affected animals needs to be performed to determine whether maternal contact increases the probability of infection. Secondly, some of the alleles are relatively rare and additional study populations from the endemic areas or from selectively bred captive white-tailed deer will be needed to increase the sample size in these genotypes. Thirdly, the interval from infection to the accumulation of detectable PrP^d levels is not known. CWD was diagnosed in deer at 8–10 months of age in this herd, establishing a minimum incubation period. The maximum incubation period cannot be identified if infection times occur outside the perinatal period (Miller & Williams, 2003). Some older adult deer had detectable PrP^d restricted to the lymphoid tissue. If the temporal pattern of PrP^d accumulation in white-tailed deer is similar to that in sheep (Hadlow *et al.*, 1982) or experimentally infected mule deer (Sigurdson *et al.*, 1999), this observation indicates that white-tailed deer can be infected as adults, as reported in mule deer (Miller & Williams, 2003) or that the incubation period can be as long as 5 years. Failure to detect PrP^d in some deer is less likely to be due to genetic differences than to the termination of the entire herd for regulatory reasons while some or all deer were in the very early stage of the disease, before PrP^d is detectable. Long-term observations of heavily exposed herds or direct oral challenge of captive deer will be needed to establish relative disease resistance in deer with the rare genotypes. However, the presence of a relatively resistant but rare genotype may not confer a selective advantage sufficient to reduce the prevalence of CWD in free-ranging herds.

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