12-2009

Genetic susceptibility to chronic wasting disease in free-ranging white-tailed deer: Complement component C1q and Prnp polymorphisms

Julie A. Blanchong
Iowa State University, julieb@iastate.edu

Dennis M. Heisey
United States Geological Survey

Kim T. Scribner
Michigan State University

Scot V. Libants
Michigan State University

Chad Johnson
University of Wisconsin - Madison
See next page for additional authors

Genetic susceptibility to chronic wasting disease in free-ranging white-tailed deer: Complement component C1q and Prnp polymorphisms

Abstract
The genetic basis of susceptibility to chronic wasting disease (CWD) in free-ranging cervids is of great interest. Association studies of disease susceptibility in free-ranging populations, however, face considerable challenges including: the need for large sample sizes when disease is rare, animals of unknown pedigree create a risk of spurious results due to population admixture, and the inability to control disease exposure or dose. We used an innovative matched case–control design and conditional logistic regression to evaluate associations between polymorphisms of complement C1q and prion protein (Prnp) genes and CWD infection in white-tailed deer from the CWD endemic area in southcentral Wisconsin. To reduce problems due to admixture or disease-risk confounding, we used neutral genetic (microsatellite) data to identify closely related CWD-positive (n = 68) and CWD-negative (n = 91) female deer to serve as matched cases and controls. Cases and controls were also matched on factors (sex, location, age) previously demonstrated to affect CWD infection risk. For Prnp, deer with at least one Serine (S) at amino acid 96 were significantly less likely to be CWD-positive relative to deer homozygous for Glycine (G). This is the first characterization of genes associated with the complement system in white-tailed deer. No tests for association between any C1q polymorphism and CWD infection were significant at $p < 0.05$. After controlling for Prnp, we found weak support for an elevated risk of CWD infection in deer with at least one Glycine (G) at amino acid 56 of the C1qC gene. While we documented numerous amino acid polymorphisms in C1q genes none appear to be strongly associated with CWD susceptibility.

Keywords
C1q, Candidate gene, Chronic wasting disease, Complement system, Conditional logistic regression, Prion, Prnp, Transmissible spongiform encephalopathy, White-tailed deer

Comments
This article is from *Infection, Genetics and Evolution* 9 (2009): 1329, doi:10.1016/j.meegid.2009.08.010.

Authors
Julie A. Blanchong, Dennis M. Heisey, Kim T. Scribner, Scot V. Libants, Chad Johnson, Judd M. Aiken, Julia A. Langenberg, and Michael D. Samuel

Infection, Genetics and Evolution 9 (2009) 1329–1335

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Genetic susceptibility to chronic wasting disease in free-ranging white-tailed deer: Complement component C1q and Prnp polymorphisms☆

Julie A. Blanchong [a,*], Dennis M. Heisey [b], Kim T. Scribner [c], Scot V. Libants [d], Chad Johnson [e], Judd M. Aiken [e], Julia A. Langenberg [f], Michael D. Samuel [g]

[a] Department of Forest and Wildlife Ecology, University of Wisconsin-Madison, Madison, WI 53706, United States
[b] U.S. Geological Survey, National Wildlife Health Center, Madison, WI 53711, United States
[c] Department of Fisheries and Wildlife and Department of Zoology, Michigan State University, East Lansing, MI 48824, United States
[d] Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824, United States
[e] Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, Madison, WI 53706, United States
[f] Wisconsin Department of Natural Resources, Madison, WI 53716, United States
[g] U. S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, University of Wisconsin-Madison, Madison, WI 53706, United States

ARTICLE INFO

Article history:
Received 12 June 2009
Received in revised form 24 August 2009
Accepted 25 August 2009
Available online 31 August 2009

Keywords:
C1q
Candidate gene
Chronic wasting disease
Complement system
Conditional logistic regression
Prion
Prnp
Transmissible spongiform encephalopathy
White-tailed deer

ABSTRACT

The genetic basis of susceptibility to chronic wasting disease (CWD) in free-ranging cervids is of great interest. Association studies of disease susceptibility in free-ranging populations, however, face considerable challenges including: the need for large sample sizes when disease is rare, animals of unknown pedigree create a risk of spurious results due to population admixture, and the inability to control disease exposure or dose. We used an innovative matched case–control design and conditional logistic regression to evaluate associations between polymorphisms of complement C1q and prion protein (Prnp) genes and CWD infection in white-tailed deer from the CWD endemic area in south-central Wisconsin. To reduce problems due to admixture or disease-risk confounding, we used neutral genetic (microsatellite) data to identify closely related CWD-positive ($n = 68$) and CWD-negative ($n = 91$) female deer to serve as matched cases and controls. Cases and controls were also matched on factors (sex, location, age) previously demonstrated to affect CWD infection risk. For Prnp, deer with at least one Serine (S) at amino acid 96 were significantly less likely to be CWD-positive relative to deer homozygous for Glycine (G). This is the first characterization of genes associated with the complement system in white-tailed deer. No tests for association between any C1q polymorphism and CWD infection were significant at $p < 0.05$. After controlling for Prnp, we found weak support for an elevated risk of CWD infection in deer with at least one Glycine (G) at amino acid 56 of the C1qC gene. While we documented numerous amino acid polymorphisms in C1q genes none appear to be strongly associated with CWD susceptibility.

1. Introduction

Chronic wasting disease (CWD) is a fatal transmissible spongiform encephalopathy (TSE) affecting free-ranging North American cervids including elk (*Cervus elaphus*), moose (*Alces alces*), mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*) (Miller et al., 2000; Baeten et al., 2007). The probable causative agent of TSEs is an abnormal isoform (PrP^{Sc}) of the naturally occurring host prion protein (PrP^{c}). The genetic basis of susceptibility and disease progression in TSE diseases is of great interest to breeding programs in domestic or captive animals (e.g., Cross and Bermester, 2002) and for predicting likely impacts of disease in free-ranging populations. Most genetic susceptibility work has focused on polymorphisms of the prion protein gene, Prnp (e.g., Johnson et al., 2003, 2006; O'Rourke et al., 1999; Perucchini et al., 2008; Wolfe et al., 2007). However, evidence from transmission disequilibrium (Hernandez-Sanchez et al., 2002) and quantitative trait loci (QTL) mapping studies (Lloyd et al., 2001; Manolakou et al., 2001) and gene inactivation, knock-out, and over-expression studies (e.g., Tamguney et al., 2008) suggest that a number of other loci could be involved in TSE pathogenesis.

In CWD, infection is typically first detectable in the peripheral lymphoid tissues. This is like scrapie, where it is hypothesized that PrP^{Sc} becomes established in the host, accumulates, and converts normal PrP^{c} protein to PrP^{Sc} before spreading to the central

☆ Note: Sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases under the accession numbers FJ775013–FJ775130.

* Corresponding author at: Department of Natural Resource Ecology and Management, 339 Science 2, Iowa State University, Ames, IA 50011, United States. Tel.: +1 515 294 9699; fax: +1 515 294 2995.
E-mail address: julieb@iastate.edu (J.A. Blanchong).

1567-1348/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2009.08.010

nervous system (Mabbot et al., 1998, 2000). These early stages of peripheral TSE infection including PrP^{Sc} invasion, dissemination, and replication likely involve activation of the complement system (Sim et al., 2007). Support for complement system involvement in early stages of TSE disease came from studies demonstrating impeded accumulation of PrP^{Sc} in lymphoid tissue and delayed spread of disease to the brain in mice deficient in C3 or C1q (Klein et al., 2001; Mabbott and Bruce, 2001). C1q recognizes and binds mouse prion that has undergone conformational modification that may mimic infectious prion conformation (Blanquet-Grossard et al., 2005; Dumestre-Perard et al., 2007), but will not bind to PrP^{c} (Dumestre-Perard et al., 2007). Additionally, Mitchell et al. (2007) provided biochemical evidence that PrP^{Sc} not only binds with C1q, but is capable of activating the classical pathway of the complement system. An important hypothesized role for the complement system in early TSE pathogenesis is facilitation of PrP^{Sc} binding and delivery to follicular dendritic cells (FDCs) by complement components C3 and C1q (Mabbott and Bruce, 2001). Complement receptors CR1 and CR2 located on FDCs recognize and trap these prion-complement complexes. Because FDCs play an important role in antigen presentation for long-term immune memory, PrP^{Sc} is hypothesized to persist and replicate on FDCs in lymphoid tissues before spreading to the central nervous system. Recently, Flores-Langarica et al. (2009) demonstrated that scrapie-associated PrP^{Sc} uptake by conventional dendritic cells (CDCs), known to be involved in early prion pathogenesis, is C1q-dependent. Therefore, complement components are potentially significant factors modulating CWD pathogenesis.

Many polymorphisms of complement system components have been characterized in humans and other mammals (e.g., Botto et al., 1990; Petry et al., 1996; Moulds et al., 2001). Some of these polymorphisms have been associated with disease susceptibility or progression (e.g., Petry et al., 1995; Messias-Reason et al., 2003; Cockburn et al., 2004). Given the apparent role of the complement system in facilitating PrP^{Sc} invasion, associations between complement gene polymorphisms and susceptibility or progression of CWD merit investigation.

Previous association studies in humans and domestic animals have demonstrated that population admixture can produce inconsistent and sometimes contradictory results (Deng, 2001; Deng et al., 2001). Admixture arises when sampled populations are composed of subpopulations with different disease-risk profiles. For example, if a true susceptibility locus with a susceptibility allele occurs at a higher frequency in one subpopulation, disease susceptibility can be spuriously associated with any other loci and alleles with higher (or lower) frequency in that subpopulation. In free-ranging populations, non-random mating can lead to population gene structure, or admixture, that can potentially confound disease association studies. A major breakthrough in addressing admixture problems was the development of the transmission disequilibrium test (TDT; Spielman et al., 1993) and later variants (Curtis, 1997; Schaid and Rowland, 1998; Spielman and Ewens, 1998). In such studies, comparisons are made among closely related individuals (e.g., non-affected parents and affected offspring), which ensures similar genetic backgrounds. TDTs are special cases of the matched case–control designs for which conditional logistic regression is the appropriate method of analysis (e.g., Waldman et al., 1998). Our study design builds on this general approach in novel ways that are generally amenable for disease association studies in free-ranging populations.

Our primary goal was to investigate the potential role of C1q polymorphisms in CWD susceptibility in free-ranging white-tailed deer from Wisconsin. As Prnp polymorphisms have been demonstrated to be associated with CWD susceptibility (O'Rourke et al., 1999, 2004; Johnson et al., 2003, 2006; Jewell et al., 2005; Fox et al., 2006; Hamir et al., 2006; Keane et al., 2008a; but see Perucchini et al., 2008), we simultaneously evaluated Prnp genotypes to account for the effect of this gene in C1q analyses and to explore potential inter-locus associations. Because, as discussed above, non-random mating and unknown pedigrees in free-ranging populations create a risk of spurious results due to admixture we used microsatellite data to identify closely related CWD-positive and CWD-negative deer to serve as matched cases and controls. We also matched cases and controls for factors (sex, location, age) previously demonstrated to affect CWD risk in our study population (Joly et al., 2006; Grear et al., 2006; Osnas et al., 2009). This innovative design allowed us to examine potential associations of C1q and Prnp polymorphisms and CWD status with relatively little risk of problems due to admixture or disease-risk confounding.

2. Materials and methods

2.1. Study area and sample selection

CWD was first detected in south-central Wisconsin in free-ranging deer harvested in 2001. We obtained samples from female white-tailed deer harvested from a ~285 km^2 area of south-central Wisconsin with the highest CWD prevalence (core-area) (5–7% of adult deer) (Joly et al., 2006, Fig. 2). For each deer, the kill-location reported by hunters was recorded as the Public Land Survey System unit "section" (2.6 km^2) and age was determined by Department of Natural Resources staff. Though there is undoubtedly some error in both the location and age data, most error is expected to be random with respect to disease status or relatedness. Retropharyngeal lymph nodes and the obex portion of the brainstem were sent to the Wisconsin Veterinary Diagnostic Laboratory (WVDL) where CWD-infection status was determined using immunohistochemistry (Johnson et al., 2006; Keane et al., 2008b). We considered a deer to be CWD-positive if PrP^{CWD} was detected in lymph nodes or obex. Female deer were sampled because they tend to have limited dispersal and spend most of their lives near their natal area (Marchinton and Hirth, 1984). Female deer in close proximity, therefore, are expected to have relatively similar exposure to prions.

We used a matched case–control design, matching controls to cases based on their relatedness at neutral markers (microsatellite loci) (e.g., Queller and Goodnight, 1989; Blouin et al., 1996). Specifically, we used a maximum likelihood analysis (program KinGroup; Konovalov et al., 2004) to identify non-infected individuals that were significantly more likely to be full siblings of a CWD-infected deer than unrelated to it. While this analysis does not guarantee that full siblings are identified, it does identify deer that are statistically significantly more likely to be very closely related than unrelated. Relatedness was determined based on the proportion of alleles the deer had in common at 14 unlinked microsatellite loci (IGF-1 Kirkpatrick, 1992; OBCAM Fries et al., 1993; CSN3, BM1225, BM6506 Bishop et al., 1994; Cervidae 1, Cervidae 2 DeWoody et al., 1995; BM4107, BM4208 Talbot et al., 1996; RT 7, RT 9, RT 23, RT 27 Wilson et al., 1997; JP15, Pemberton unpublished) relative to population allele frequencies generated from ~1400 female deer in the study area (Grear, 2006). Average relatedness among female deer in the study area was low. Even for deer harvested within the same section (2.6 km^2) average relatedness was only $r = 0.004$ (Grear, 2006). Relatedness among female deer declined with increasing distance separating animals such that average relatedness was not significantly different from $r = 0$ for deer separated by roughly 3.2 km or greater (Grear, 2006).

For each CWD-infected deer (case, $n = 68$) we selected at least one non-infected deer (control, $n = 91$) that met the above relatedness criterion. In addition, because prevalence, and thus disease exposure, varies by spatial location and age (Grear et al.,

2006; Joly et al., 2006; Osnas et al., 2009) each CWD-infected deer was matched with a non-infected deer harvested within a 5.6 km radius of the infected animal's location, and when possible, of similar age. We used this spatially explicit and relatedness-based matched case control design to reduce potential confounding due to admixture and to increase statistical power.

2.2. C1q gene sequencing

We sequenced the globular head regions of complement 1 subcomponent q polypeptides alpha, beta, and gamma (C1qA, C1qB, C1qC) in CWD-positive and CWD-negative deer. C1q is composed of 18 polypeptide chains: six A-chains, six B-chains, and six C-chains. Each chain contains a collagen-like region located near the N-terminal and a C-terminal globular region. The globular heads of C1q are responsible for the recognition of a large variety of ligands including prions (Gaboriauld et al., 2003, 2004). C1qA, B, and C are relatively small (245–253 amino acids), they are encoded within a small portion of the chromosome (between 2953 base pairs (bp), 8557 bp and 4480 bp for C1qA, B, and C respectively), and the entire protein is encoded within two exons for each gene. The globular head region is contained within a single exon for each of the coding regions for C1qA, B, and C.

Primers were designed from genomic DNA sequences predicted to code for the C1q genes based on the genome of the domestic cow (*Bos taurus*). Primers for each C1q gene were designed from aligned *B. taurus* genomic and mRNA sequences to flank the predicted coding region for the globular domain of each C1q polypeptide. We confirmed the position of each globular head alignment with the better-characterized human C1q polypeptide sequence. Accession numbers for each C1q polypeptide in *B. taurus* and humans, PCR sequencing primers for each C1q polypeptide in white-tailed deer, and accession numbers for sequences identified in this study are provided in Table 1.

Amplification reaction conditions for each C1q gene were as follows: 20 ng of genomic DNA, 200 μM dNTPs, 2.5 mM $MgCl_2$, 2 pmol each primer, and 1 U ChromaTAQ (Denville Scientific, Inc.) in ChromaTAQ Buffer in a total volume of 50 μL. During 30 cycles of amplification, primers annealed at 50 °C for C1qA and C1qB and 60 °C for C1qC. This amplification generated a 672 bp fragment of C1qA, a 700 bp fragment of C1qB, and a 900 bp fragment of C1qC. PCR products were purified using Millipore Montage Centrifugal PCR filters or the Qiagen Qiaquick PCR Purification Kit. 20 ng of PCR product was used as template in the sequencing reactions. Sequences of C1qA were generated using the F2A primer, sequences of C1qB were generated using the F11B primer, and sequences of C1qC were generated using the BosQC2R primer (Table 1).

For each C1q gene, PCR products were sequenced using an ABI Prism 3700 DNA Analyzer at the Research Technology Support Facility at Michigan State University. We trimmed sequences for quality ($Q > 20$), aligned them, and used the chromatogram data to verify each polymorphic site. To determine the reading frame, sample sequences were analyzed by a translated BLAST search (blastx and tblastx). The reading frame used for analysis of the C1qA globular head sequences was from base positions 1 through 423 on the plus strand. This reading frame was determined from the high-scoring alignment with the predicted *B. taurus* C1qA sequence (GenBank Accession number NP_001014945). The reading frame used for analysis of C1qB globular head sequences was from base position 9 through 537 on the minus strand. This was determined from the high-scoring alignment with the predicted *B. taurus* C1qB sequence (GenBank Accession number XP_874787). The reading frame used for analysis of C1qC globular head sequences was from base positions 2 through 508 on the plus strand. This was determined from the high-scoring alignment with the predicted *B. taurus* C1qC sequence (GenBank Accession number XP_587039).

We performed translations and alignments on all unique sequences using MacVector. We grouped representative genotypes for analysis using the program Collapse v1.1 (David Posada, 1999). Sequences that contained double-peaks of approximately equal height, indicating the presence of two different bases, were coded with International Union of Pure and Applied Chemistry (IUPAC) degeneracy codes and treated as polymorphisms. We replaced each co-termination in the sequence, M (a/c), R (a/g), W (a/t), S (c/g), and Y(c/t), with each of the two possible nucleotides and translated them. The two resulting amino acid sequences were aligned to one another to determine if a co-termination resulted in an amino acid substitution in the predicted protein.

2.3. Prion gene sequencing

We sequenced the Prnp gene in all CWD-positive and CWD-negative deer samples as described in detail in Johnson et al. (2003) to verify previously observed patterns of association between Prnp polymorphisms and CWD infection in Wisconsin deer (Johnson et al., 2003, 2006) and to investigate potential interactions between Prnp and C1q gene polymorphisms and CWD infection.

2.4. Statistical analyses of association between C1q and Prnp genes and CWD infection

We considered three a priori nested models of gene association with CWD infection. The first two models (I, II) were specific to each amino acid variable site (AAVS). Model I was a simple amino acid presence/absence model that did not differentiate between heterozygous and homozygous individuals. Model I was applied to all AAVSs within each of the four genes (Prnp, C1qA, C1qB, C1qC). Model II considered all observed amino acid dyads, within each AAVS. In addition to being sensitive to heterozygosity, this model was also sensitive to dose effects; differences between single dose (heterozygous) and double dose (homozygous) states. Models I and II were applied on an AAVS-specific basis. Because of the importance of protein conformation in TSE diseases, it is plausible that AAVSs might interact. Model III provided an extension of

Table 1
Accession numbers used in the design of primers to sequence C1q genes in white-tailed deer, primer pairs used to amplify C1q genes in this study, and accession numbers of sequences in this study.

Gene	Accession number *Bos taurus* genomic DNA	Accession number *Bos taurus* mRNA	Accession number human mRNA	Primer pairs White-tailed deer	Accession number White-tailed deer
C1qA	NC_007300	NM_001014945	NM_015991	F2A (5′-TTA AAG GAG ACC AGG GCG AC-3′) B5A (5′-GAC ATG ATC TCC ATT TAG GC-3′)	FJ775126–FJ775130
C1qB	NC_007300	XM_869694	NM_000491	F11B (5′-TGG AGC CCT ACT GTG TGC TTT G-3′) R11B (5′-CGT TAG CCA CCT CAA CGA GAA TG-3′)	FJ775013–FJ775065
C1qC	NC_931001	XM_587039	NM_172369	F1-CF (5′-GGG AAT CGC TTT TTA GGG AAT TGG-3′) BosQC2R (5′-TCA CTG CCT TCC CAC CTG CTC TCT C-3′)	FJ775066–FJ775125

Model II that considered all amino acid dyads across all AAVSs within a gene. Thus, Model III considered the genotype across all AAVSs within a gene; hence one Model III analysis per gene.

For C1qA, B, and C, we also controlled for the effects of Prnp. Specifically, we analyzed a special a priori case of Model I where we added an indicator of the presence or absence of Serine (S) at amino acid 96 of the Prnp gene because of the support for the importance of this amino acid position in CWD susceptibility and progression (Johnson et al., 2006; Keane et al., 2008a). We report these results with the Model I results.

Data were analyzed using conditional logistic regression (Kleinbaum et al., 1982) by matching at least one control (disease-negative deer) to each case (disease-positive deer) on the basis of relatedness, harvest location, and age. This design reduced the risk of obtaining spurious associations due to population admixture or disease-risk confounding. Major distinctions from the typical contingency table (cohort) approach used in previous CWD studies are that case–control analyses are performed within the matched sets which improves design efficiency (smaller sample sizes required) for investigation of rare diseases where controls are more common than cases (Kleinbaum et al., 1982) and provides control of other factors that influence disease risk. Conditional logistic regression was performed with SAS PROC PHREG (SAS Institute Inc., Cary, NC). All reported *p*-values were based on likelihood ratio statistics except in the case of Model I analyses where Wald statistics were used. We report the odds ratios (OR) and 95% confidence intervals (95% CI) for the amino acid effects for Model I.

Because other studies have reported a relationship between disease progression (stage) and Prnp polymorphisms (e.g., Johnson et al., 2006; Keane et al., 2008a), we tested for associations between early disease stage (lymph node infection only) versus later stage (lymph node and brain infection) and C1q and Prnp polymorphisms using Fisher's exact test (two-sided). We performed these analyses on a gene-wise (Model III) basis.

We also tested for Hardy–Weinberg (HW) equilibrium at each variable site for each gene for all animals combined and for CWD-positive and CWD-negative animals separately. Departures from HW equilibrium, especially in the positive group, may give evidence for associations between genotypes and disease status.

3. Results

3.1. C1Q polymorphism

No deer were heterozygous at the nucleotide level across the 423 bp region of C1qA that we sequenced. Heterozygosity at the nucleotide level was fairly common, however, in both the 542 bp C1qB and 508 bp C1qC globular head regions though most substitutions were silent when examined at the amino acid (AA)

Table 2
Disease association with the presence or absence of a particular amino acid at a specific amino acid variable site (corresponds to Model I in text).

Locus	AAVS[a]	AA	Number positive	Number positive	*p*-Value	Prnp-adjusted	Odds ratio	95% CI
Prnp	95	Q	65	72	na	na	na	na
		No Q	0	0				
		H	0	1	0.994	na	0	0–∞
		No H	65	71				
	96	G	64	68	0.310	na	3.1	0.33–29.9
		No G	1	4				
		S	9	31	0.002	na	0.22	0.08–0.59
		No S	56	41				
C1qA	4	N	60	79	0.896	0.995	0.82	0.04–18.4
		No N	1	2				
		H	1	2	0.896	0.995	1.23	0.05–27.6
		No H	60	79				
	118	K	59	78	0.239	0.995	0	0–∞
		No K	2	3				
		E	2	3	0.239	0.995	∞	0–∞
		No E	59	78				
C1qB	72	A	64	80	na	na	na	na
		No A	0	0				
		P	0	2	0.994	0.994	0	0–∞
		No P	64	78				
	164	I	63	80	0.994	0.994	0	0–∞
		No I	1	0				
		F	1	0	0.994	0.994	∞	0–∞
		No F	63	80				
	170	L	64	80	na	na	na	na
		No L	0	0				
		H	1	1	0.809	0.282	1.4	0.08–25.0
		No H	63	79				
C1qC	30	E	67	82	na	na	na	na
		No E	0	0				
		V	4	1	0.341	0.994	3.00	0.30–30.2
		No V	63	81				
	56	G	45	53	0.205	0.083	1.82	0.71–4.71
		No G	22	29				
		S	33	39	0.567	0.530	0.74	0.33–1.65
		No S	34	43				

[a] Amino acid variable site.

Table 3
Disease association with the presence or absence of a particular amino acid pair (genotype) at a specific amino acid variable site (corresponds to Model II in text).

Gene	AAVS[a]	Genotypes	Number positive	Number negative	*p*-Value
Prnp	95	QQ	65	71	0.239
		QH	0	1	
	96	GG	56	41	0.002
		GS	8	27	
		SS	1	4	
C1qA	4	NN	60	79	0.896
		HH	1	2	
	118	KK	59	78	0.239
		EE	2	3	
C1qB	72	AA	64	78	0.096
		AP	0	2	
	164	II	63	80	0.138
		FF	1	0	
	170	LL	63	79	0.810
		LH	1	1	
C1qC	30	EE	63	81	0.306
		EV	4	1	
	56	GG	34	43	0.427
		GS	11	10	
		SS	22	29	

[a] Amino acid variable site.

level. The reason for differences in heterozygosity among the C1q genes is unknown.

For C1qA, we sequenced 142 deer ($n = 61$ case, $n = 81$ control). We identified five unique nucleotide sequences producing variable sites at AAs 4 and 118 (Tables 2 and 3) resulting in three different predicted AA sequences each 140 AA long (Table 4). For C1qB, we sequenced 144 deer ($n = 64$ case, $n = 80$ control). We identified 53 unique nucleotide sequences for C1qB producing variable sites at AAs 72, 164, and 170 (Tables 2 and 3) resulting in four different predicted AA sequences (176 AA) (Table 4). For C1qC, we sequenced 149 deer ($n = 67$ case, $n = 82$ control). We identified 60 unique C1qC nucleotide sequences producing variable sites at AAs 30 and 56 (Tables 2 and 3) resulting in four different predicted AA sequences (169AA) (Table 4).

Table 4
Disease association for unique genotypes across all amino acid variable sites for each gene (corresponds to Model III in text).

Locus	AAVS[a]	Unique genotypes	Number positive	Number negative	*p*-Value
Prnp	95, 96	QQ, GG	56	40	0.001
		QQ, GS	8	27	
		QQ, SS	1	4	
		QH, GG	0	1	
C1qA	4, 118	NN, KK	59	78	0.333
		NN, EE	1	1	
		HH, EE	1	2	
C1qB	72, 164, 170	AA, II, LL	62	77	0.170
		AA, II, LH	1	1	
		AA, FF,LL	1	0	
		AP, II, LL	0	2	
C1qC	30, 56	EE, GG	33	43	0.426
		EE, GS	9	9	
		EE, SS	21	29	
		EV, GG	1	0	
		EV, GS	2	1	
		EV, SS	1	0	

[a] Amino acid variable site.

3.2. Prnp polymorphism

For Prnp, we sequenced 137 deer ($n = 65$ case, $n = 72$ control). We identified variable sites at AAs 95 and 96 (Tables 2 and 3) resulting in four different AA sequences (Table 4).

3.3. Associations between genotype and CWD infection

Association between Prnp polymorphisms and CWD infection using conditional logistic regression analyses on matched cases and controls confirmed those documented by Johnson et al. (2006). Specifically, deer with at least one Serine (S) at AA 96 were significantly less likely (OR = 0.22, 95% CI = 0.08–0.59, $p = 0.002$, Table 2) to be CWD-positive (cases) relative to animals homozygous for Glycine (G). There was no evidence for deviations from HW equilibrium at any of the Prnp AAVSs ($p > 0.3$).

No tests for association between any C1q polymorphisms and CWD infection were significant at $p < 0.05$ (Tables 2–4). The strongest statistical support for a C1q association was an increased risk of CWD infection for deer with a Glycine (G) at AA 56 of the C1qC gene after adjusting for the presence/absence of Serine (S) at AA 96 of the Prnp gene (OR = 2.82, 95% CI = 0.85–9.32, $p = 0.083$, Table 2). The apparently large odds ratio (OR = 3, Table 2) for the presence/absence of Valine (V) at AA 30 of the C1qC gene does not approach statistical significance ($p = 0.341$), likely because of the rarity of this polymorphism. There was also evidence for deviations from HW equilibrium (heterozygote deficiency; $p < 0.001$) at several C1q AAVSs (AA 4 C1qA; AAs 72, 164 C1qB; AA 56 C1qC) in both CWD-positive and CWD-negative animals.

All Fisher's exact tests for association between C1q or Prnp and disease stage were non-significant (all $p > 0.05$), although C1qA was borderline at $p = 0.055$. Of the 56 CWD-positive animals for which we had data on disease stage, none of the 44 later stage deer (both lymph node and brain infection) were homozygous for Asparagine (NN), Glutamic Acid (EE) or Histidine (HH), Glutamic Acid (EE) while of the 12 early stage deer (lymph node infection only), one each were NN, EE and HH, EE.

4. Discussion

Polymorphisms in complement genes can significantly affect disease pathogenesis (Maeurer et al., 1992; Petry et al., 1995; Messias-Reason et al., 2003; Cockburn et al., 2004). For example, in humans, mutations in C1q genes have been demonstrated to modulate arthritis symptoms (Maeurer et al., 1992) and are associated with systemic lupus erythematosus (Petry et al., 1995). The complement system appears to play an important role in early TSE pathogenesis (Mabbott and Bruce, 2001; Flores-Langarica et al., 2009). Using intraperitoneal inoculations of low dilutions of PrP^{Sc} in mice, Klein et al. (2001) discovered that while 100% of control animals were susceptible to infection, none of the C1q deficient animals became infected (Klein et al., 2001, Table 1). Besides complete gene deficiency, polymorphisms could influence the ability of C1q to bind and deliver PrP^{Sc} to follicular dendritic cells or to participate in PrP^{Sc} uptake in common dendritic cells, possibly affecting the rate of PrP^{Sc} invasion and the progression of disease. These data are the first characterization of genes associated with the complement system in white-tailed deer and the first assessment of the role of complement gene polymorphism in TSE susceptibility. While we detected numerous amino acid polymorphisms in the globular head regions of C1qA, B, and C genes, most were quite rare and none were as strongly associated with CWD infection as the polymorphism at AA 96 in the Prnp gene.

The relatively intensive harvest of deer in our study population (Blanchong et al., 2006) has led to a young age structure in which

deer appear to successfully breed even when very young (Blanchong et al., unpublished). Because young animals are at significantly lower risk of CWD infection than older animals (Grear et al., 2006; Osnas et al., 2009) many deer are likely to reproduce before they become infected. Reproduction prior to infection will reduce the effectiveness of disease as a selective agent. In addition, because clinical symptoms of CWD in infected animals take several months to years to appear (Williams, 2005), it is very likely that infected animals produce offspring prior to dying from CWD further reducing selection pressure and limiting gene frequency changes at disease-associated loci over time. In addition to the likelihood that deer breed before infection or clinical signs of disease, CWD prevalence in our study area is fairly low further limiting the opportunity for selection for disease-resistance to shift gene frequencies. As such, weak associations between C1q polymorphisms and CWD susceptibility may not be detectable in this population. Associations between C1q polymorphisms and CWD susceptibility might be detectable in areas where the disease has been established for a longer time, disease is at higher prevalence, and age structure is more normally distributed such that younger deer may be less likely to breed (e.g., Colorado or Wyoming).

Identifying the genetic basis for heterogeneity in disease susceptibility or progression can improve our understanding of individual variation in disease susceptibility in both free-ranging and captive populations. What this individual variation in disease susceptibility means for the trajectory of disease in a population, however, is not straightforward. For example, the greater, but not complete, resistance to CWD in deer with at least one Serine (S) at amino acid 96 of the Prnp gene appears to be associated with slower progression of disease (e.g., Johnson et al., 2006; Keane et al., 2008a). If slower disease progression results in longer-lived, infected deer with longer periods of infectiousness, resistance may lead to increased disease transmission rates, higher prion concentrations in the environment, and increased prevalence, as has been observed in some captive deer herds (Miller et al., 2006; Keane et al., 2008a). Alternatively, if the slower progression of disease in resistant deer is not associated with longer periods of infectiousness, but might instead indicate a higher dose of PrP^{CWD} is required for infection, transmission rates in the population could decline especially if, as in Wisconsin, deer suffer high rates of mortality from other sources (e.g., hunting). Clearly, determining the relationship between genetic susceptibility to infection, dose requirements, disease progression, and the period of PrP^{CWD} infectiousness are key components for understanding the consequences of CWD to free-ranging populations.

Acknowledgements

We thank Cathy Cullingham, Bryan Richards and two anonymous reviewers for comments that improved the quality of the manuscript. We also thank the many volunteers and Wisconsin Department of Natural Resources staff who collected deer tissue samples and the hunters who participated in CWD management. Funding was provided by the US Geological Survey-National Wildlife Health Center. Mention of trade names or commercial products does not imply an endorsement by the U.S. Government.

References

Baeten, L.A., Powers, B.E., Jewell, J.E., Spraker, T.R., Miller, M.W., 2007. A natural case of chronic wasting disease in a free-ranging moose (*Alces alces shirasi*). J. Wildl. Dis. 43, 309–314.

Bishop, M.P., Kappes, S.M., Keele, J.W., Stone, R.T., Sunden, S.F., Hawkins, G.A., Toldo, S.S., Fries, R., Grosz, M.D., Yoo, J., Beattie, C.W., 1994. A genetic linkage map for cattle. Genetics 136, 619–639.

Blanchong, J.A., Joly, D.O., Samuel, M.D., Langenberg, J.A., Rolley, R.E., Sausen, J.E., 2006. White-tailed deer harvest from the chronic wasting disease eradication zone in south-central Wisconsin. Wild. Soc. Bull. 34, 725–731.

Blanquet-Grossard, F.N., Thielens, M., Vendrely, C., Jamin, M., Arlaud, G.J., 2005. Complement protein C1q recognizes conformationally modified form of the prion protein. Biochemistry 44, 4349–4356.

Blouin, M.S., Parsons, M., Lacaille, V., Lotz, S., 1996. Use of microsatellite loci to classify individuals by relatedness. Mol. Ecol. 5, 393–401.

Botto, M., Fong, K.Y., So, A.K., Koch, C., Walport, M.J., 1990. Molecular basis of polymorphisms of human complement component C3. J. Exp. Med. 172, 1011–1017.

Cockburn, I.A., Mackinnon, M.J., O'Donnell, A., Allen, S.J., Moulds, J.M., Baisor, M., Bockarie, M., Reeder, J.C., Rowe, J.A., 2004. A human complement receptor 1 polymorphism that reduces *Plasmodium falciparum* rosetting confers protection against severe malaria. Proc. Natl. Acad. Sci. U.S.A. 101, 272–277.

Cross, D., Bermester, J.K., 2002. A genetic approach to the problem of chronic wasting disease in Wisconsin. Wis. Med. J. 101, 25–27.

Curtis, D., 1997. Use of siblings as controls in case–control association studies. Ann. Hum. Genet. 61, 319–333.

Deng, H.W., 2001. Population admixture may appear to mask, change or reverse genetic effects of genes underlying complex traits. Genetics 159, 1319–1323.

Deng, H.W., Chen, W.M., Recker, R.R., 2001. Population admixture: detection by Hardy–Weinberg test and its quantitative effects on linkage-disequilibrium methods for localizing genes underlying complex traits. Genetics 157, 885–897.

DeWoody, J.A., Honeycutt, R.L., Skow, L.C., 1995. Microsatellite markers in white-tailed deer. J. Heredity 86, 317–319.

Dumestre-Perard, C., Osmundson, J., Lemaire-Vielle, C., Thielens, N., Grives, A., Favier, B., Csopaki, F., Jamin, M., Gagnon, J., Cesbron, J.-Y., 2007. Activation of classical pathway of complement cascade by soluble oligomers of prion. Cell Microbiol. 9, 2870–2879.

Flores-Langarica, A., Sebti, Y., Mitchell, D.A., Sim, R.B., MacPherson, G.G., 2009. Scrapie pathogenesis: the role of complement C1q in scrapie agent uptake by conventional dendritic cells. J. Immunol. 182, 1305–1313.

Fox, K.A., Jewell, J.E., Williams, E.S., Miller, M.W., 2006. Patterns of PrP^{CWD} accumulation during the course of chronic wasting disease infection in orally inoculated mule deer *Odocoileus hemionus*. J. Gen. Virol. 87, 3451–3461.

Fries, R., Eggen, A., Womack, J.E., 1993. The bovine genome map. Mamm. Genome 4, 405–408.

Gaboriauld, C., Juanhulx, J., Gruez, A., Lacroix, M., Darnault, C., Pignol, D., Verger, D., Fontecilla-Camps, J.C., Arlaud, G.J., 2003. The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. J. Biol. Chem. 278, 46974–46982.

Gaboriauld, C., Thielens, N.M., Gregory, L.A., Rossi, V., Fontecilla-Campls, J.C., Arlaud, G.J., 2004. Structure and activation of the C1 complex of complement: unraveling the puzzle. Trends Immunol. 25, 368–373.

Grear, D.A., 2006. Masters thesis. University of Wisconsin-Madison.

Grear, D.A., Samuel, M.D., Langenberg, J.A., Keane, D., 2006. Demographic patterns and harvest vulnerability of chronic wasting disease infected white-tailed deer in Wisconsin. J. Wildl. Manage. 70, 546–553.

Hamir, A.N., Gidlewski, T., Spraker, T.R., Miller, J.M., Creekmore, L., Crocheck, M., Cline, T., O'Rourke, K.I., 2006. Preliminary observations of genetic susceptibility of elk (*Cervus elaphus nelsoni*) to chronic wasting disease by experimental inoculation. J. Vet. Diagn. Invest. 18, 110–114.

Hernandez-Sanchez, J., Waddington, D., Wiener, P., Haley, C.S., Williams, J.L., 2002. Genome-wide search for markers associated with bovine spongiform encephalopathy. Mamm. Genome 13, 164–168.

Jewell, J.E., Conner, M.M., Wolfe, L.L., Miller, M.W., Williams, E.S., 2005. Low frequency of PrP genotype 225SF among free-ranging mule deer (*Odocoileus hemionus*) with chronic wasting disease. J. Gen. Virol. 86, 2127–2134.

Johnson, C., Johnson, J., Clayton, M., McKenzie, D., Aiken, J., 2003. Prion protein gene heterogeneity in free-ranging white-tailed deer within the chronic wasting disease affected region of Wisconsin. J. Wildl. Dis. 39, 576–581.

Johnson, C., Johnson, J., Vanderloo, J.P., Keane, D., Aiken, J.M., McKenzie, D., 2006. Prion protein polymorphisms in white-tailed deer influence susceptibility to chronic wasting disease. J. Gen. Virol. 87, 2109–2114.

Joly, D.O., Samuel, M.D., Langenberg, J.A., Blanchong, J.A., Batha, C.A., Rolley, R.E., Keane, D.P., Ribic, C.A., 2006. Spatial epidemiology of chronic wasting disease in Wisconsin white-tailed deer. J. Wildl. Dis. 42, 578–588.

Keane, D.P., Barr, D.J., Bochsler, P.N., Hall, S.M., Gidlewski, T., O'Rourke, K.I., Spraker, T.R., Samuel, M.D., 2008a. Chronic wasting disease in a Wisconsin white-tailed deer farm. J. Vet. Diagn. Invest. 20, 698–703.

Keane, D.P., Barr, D.J., Keller, J.E., Hall, S.M., Langenberg, J.A., Bochsler, P.N., 2008b. Comparison of retropharyngeal lymph node and obex region of the brainstem in detection of chronic wasting disease in white-tailed deer (*Odocoileus virginianus*). J. Vet. Diagn. Invest. 20, 58–60.

Kirkpatrick, B.W., 1992. Identification of a conserved microsatellite site in the porcine and bovine insulin-like growth factor-I gene 5′ flank. Anim. Genet. 23, 543–548.

Klein, M.A., Kaeser, P.S., Schwarz, S., Weyd, H., Xenarios, I., Zinkernagel, R.M., Carroll, M.C., Verbeek, S., Botto, M., Walport, M.J., Molina, H., Kalinke, U., Acha-Orbea, H., Aguzzi, A., 2001. Complement facilitates early prion pathogenesis. Nat. Med. 7, 488–492.

Kleinbaum, D.G., Kupper, L.L., Morgenstern, H., 1982. Epidemiological Research. Van Nostrand Reinhold Publishers, New York.

Konovalov, D.A., Manning, C., Henshaw, M.T., 2004. KINGROUP: a program for pedigree relationship reconstruction and kin group assignments using genetic markers. Mol. Ecol. Notes 4, 779–782.

Lloyd, S.E., Onwuazor, O.N., Beck, J.A., Mallinson, G., Farrall, M., Targonski, P., Collinge, J., Fisher, E.M., 2001. Identification of multiple quantitative trait loci

linked to prion disease incubation period in mice. Proc. Natl. Acad. Sci. U.S.A. 98, 6279–6283.
Mabbot, N.A., Farquhar, C.F., Brown, K.L., Bruce, M.E., 1998. Involvement of the immune system in TSE pathogenesis. Immunol. Today 19, 201–203.
Mabbot, N.A., Mackay, F., Minns, F., Bruce, M.E., 2000. Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie. Nat. Med. 6, 719–720.
Mabbott, N.A., Bruce, M.E., 2001. The immunobiology of TSE diseases. J. Gen. Virol. 82, 2307–2318.
Maeurer, M.J., Trinder, P.K.E., Loos, M., 1992. Arthritis-regulating determinants on the collagen-like complement component C1q. Immunol. Today 13, 278–279.
Manolakou, K., Beaton, J., McConnell, I., Farquar, C., Manson, J., Hastie, N.D., Bruce, M., Jackson, I.J., 2001. Genetic and environmental factors modify bovine spongiform encephalopathy incubation period in mice. Proc. Natl. Acad. Sci. U.S.A. 98, 7402–7407.
Marchinton, R.L., Hirth, D.H., 1984. Behavior. In: Halls, L.K. (Ed.), White-tailed Deer Ecology and Management. Stackpole, Harrisburg, pp. 129–168.
Messias-Reason, I.L., Urbanetz, L., Pereira da Cunha, C., 2003. Complement C3 F and BF S allotypes are risk factors for Chagas disease cardiomyopathy. Tissue Antigens 62, 308–312.
Miller, M.W., Williams, E.S., McCarty, C.W., Spraker, T.R., Kreeger, T.J., Larsen, C.T., Thorne, E.T., 2000. Epizootiology of chronic wasting disease in free-ranging cervids in Colorado and Wyoming. J. Wildl. Dis. 36, 676–690.
Miller, M.W., Hobbs, N.T., Tavener, S.J., 2006. Dynamics of prion disease transmission in mule deer. Ecol. Appl. 16, 2208–2214.
Mitchell, D.A., Kirby, L., Paulin, S.M., Villers, C.L., Sim, R.B., 2007. Prion protein activates and fixes complement directly via the classical pathway: implications for the mechanism of scrapie agent propagation in lymphoid tissue. Mol. Immunol. 44, 2997–3004.
Moulds, J.M., Zimmerman, P.A., Doumbo, O.K., Kassambara, L., Sagara, I., Diallo, D.A., Atkinson, J.P., Krych-Goldberg, M., Hauhart, R.E., Hourcade, D.E., McNamara, D.T., Birmingham, D.J., Rowe, J.A., Moulds, J.J., Miller, L.H., 2001. Molecular identification of Knops blood group polymorphisms found in long homologous region D of complement receptor 1. Blood 97, 2879–2885.
O'Rourke, K.I., Besser, T.E., Miller, M.W., Cline, T.F., Spraker, T.R., Jenny, A.L., Wild, M.A., Zebarth, G.L., Williams, E.S., 1999. PrP genotypes of captive and free-ranging Rocky Mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease. J. Gen. Virol. 80, 2765–2769.
O'Rourke, K.I., Spraker, T.R., Hamburg, L.K., Besser, T.E., Brayton, K.A., Knowles, D.P., 2004. Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. J. Gen. Virol. 85, 1339–1346.
Osnas, E.E., Heisey, D.M., Rolley, R.E., Samuel, M.D., 2009. Spatial and temporal patterns of chronic wasting disease: fine-scale mapping of a wildlife epidemic in Wisconsin. Ecol. Appl. 19, 1311–1322.
Perucchini, M., Griffin, K., Miller, M.W., Goldmann, W., 2008. PrP genotypes of free-ranging wapiti (*Cervus elaphus nelsoni*) with chronic wasting disease. J. Gen. Virol. 89, 1324–1328.
Petry, F., Le, D.T., Kirschfink, M., Loos, M., 1995. Nonsense and missense mutations in the structural genes of complement component C1q A-chains and C-chains are linked with 2 different types of complete selective C1q deficiencies. J. Immunol. 155, 4734–4738.
Petry, F., McClive, P.J., Botto, M., Morley, B.J., Morahan, G., Loos, M., 1996. The mouse C1q genes are clustered on chromosome 4 and show conservation of gene organization. Immunogenetics 43, 370–376.
Queller, D.C., Goodnight, K.F., 1989. Estimating relatedness using genetic markers. Evol. Int. J. Org. Evol. 43, 258–275.
Schaid, D.J., Rowland, C., 1998. Use of parents, sibs and unrelated controls for detection of associations between genetic markers and disease. Am. J. Hum. Genet. 63, 1492–1506.
Sim, R.B., Kishore, U., Villiers, C.L., Marche, P.N., Mitchell, D.A., 2007. C1q binding and complement activation by prions and amyloids. Immunobiology 212, 355–362.
Spielman, R.S., Ewens, W.J., 1998. A sibship test for linkage in the presence of association: the sib transmission/disequilibrium test. Am. J. Hum. Genet. 62, 450–458.
Spielman, R.S., McGinnnis, R.E., Ewens, W.J., 1993. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (DDM). Am. J. Hum. Genet. 52, 506–516.
Talbot, J., Haigh, J., Plante, Y., 1996. A parentage evaluation test in North American elk (wapati) using microsatellites of ovine and bovine origin. Anim. Genet. 27, 117–119.
Tamguney, G., Giles, K., Gliden, D.V., Lessard, P., Wille, H., Tremblay, P., Groth, D.F., Yehiely, F., Korth, C., Moore, R.C., Tatzelt, J., Rubinstein, E., Boucheix, C., Yang, X., Stanley, P., Lisanti, M.P., Dwek, R.A., Rudd, P.M., Moskovitz, J., Epstein, C.J., Dawson Cruz, T., Kuziel, W.A., Maeda, N., Sap, J., Hsiao Ashe, K., Carlson, G.A., Tesseur, I., Wyss-Coray, T., Mucke, L., Weisgraber, K.H., Mahley, R.W., Cohen, F.E., Prusiner, S.B., 2008. Genes contributing to prion pathogenesis. J. Gen. Virol. 89, 1777–1788.
Waldman, I.D., Robinson, B.F., Rowe, D.C., 1998. A logistic regression based extension of the TDT for continuous and categorical traits. Ann. Hum. Genet. 63, 329–340.
Williams, E.S., 2005. Chronic wasting disease. Vet. Pathol. 42, 530–549.
Wilson, G.A., Strobeck, C., Wu, L., Coffin, J.W., 1997. Characterization of microsatellite loci in caribou, *Rangifer tarandus*, and their use in other artiodactyls. Mol. Ecol. 6, 697–699.
Wolfe, L.L., Spraker, T.R., Gonzalez, L., Dagleish, M.P., Sirochman, T.M., Brown, J.C., Jeffrey, M., Miller, M.W., 2007. PrPCWD in rectal lymphoid tissue of deer (*Odocoileus* spp.). J. Gen. Virol. 88, 2078–2082.