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Bison *PRNP* genotyping and potential association with *Brucella* spp. seroprevalence

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Summary

The implication that host cellular prion protein (PrP^C) may function as a cell surface receptor and/or portal protein for *Brucella abortus* in mice prompted an evaluation of nucleotide and amino acid variation within exon 3 of the prion protein gene (*PRNP*) for six US bison populations. A non-synonymous single nucleotide polymorphism (T50C), resulting in the predicted amino acid replacement M17T (Met → Thr), was identified in each population. To date, no variation (T50; Met) has been detected at the corresponding exon 3 nucleotide and/or amino acid position for domestic cattle. Notably, 80% (20 of 25) of the Yellowstone National Park bison possessing the C/C genotype were *Brucella* spp. seropositive, representing a significant ($P = 0.021$) association between seropositivity and the C/C genotypic class. Moreover, significant differences in the distribution of *PRNP* exon 3 alleles and genotypes were detected between Yellowstone National Park bison and three bison populations that were either founded from seronegative stock or previously subjected to test-and-slaughter management to eradicate brucellosis. Unlike domestic cattle, no indel polymorphisms were detected within the corresponding regions of the putative bison *PRNP* promoter, intron 1, octapeptide repeat region or 3′-untranslated region for any population examined. This study provides the first evidence of a potential association between nucleotide variation within *PRNP* exon 3 and the presence of *Brucella* spp. antibodies in bison, implicating PrP^C in the natural resistance of bison to brucellosis infection.

Keywords bison, brucellosis, natural resistance, *PRNP*.

Introduction

Brucella abortus is a gram-negative facultative intracellular pathogen associated with brucellosis infection in mammals, including undulant fever in humans and abortion and/or infertility in many domestic and wild animals (Acha & Szylres 1980). Although classical breeding studies in domestic cattle (*Bos taurus*) have indicated that natural resistance to *B. abortus* is controlled by two or more genes (Templeton *et al.* 1988), few genes have been investigated and only one, *NRAMP1*, has been implicated in natural resistance to brucellosis in domestic cattle (Feng *et al.* 1996). *NRAMP1* is an integral protein hypothesized to be involved in phagosome acidification and/or phagosome–lysosome fusion processes within macrophages and has been associated with infectious and autoimmune diseases (Blackwell *et al.* 2001). To date, no association between *NRAMP1* and natural resistance to brucellosis has been demonstrated in American bison (*Bison bison*), a member of the closely related sister genus to *Bos*.

Recently, *B. abortus* heat shock protein 60 was demonstrated to interact with host-encoded cellular prion protein (PrP^C), aiding in the establishment of infection into mouse macrophages and implicating PrP^C as a potential receptor for *B. abortus* (Watarai *et al.* 2003; Watarai 2004). While no consensus regarding the physiological function of PrP^C has been reached to date (Collinge 2001; Aguzzi & Hardt 2003), it may function as a cell-surface receptor for signal transduction (Mouillet-Richard *et al.* 2000). Moreover, the rapid cycling of PrP^C between the cell surface and the early endosome via clathrin-coated vesicles is a physiological process shared by many other cell-surface receptors (Shyng *et al.* 1994). Notably, amino acid polymorphisms associated with the *PRNP* gene are known to influence the phenotypic

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expression of scrapie in sheep and goats (Belt *et al.* 1995; Billinis *et al.* 2002), Creutzfeld Jakob disease, Gerstman Sträussler Scheinker disease, and Kuru in humans (Collinge 2001), and chronic wasting disease in deer and elk (O'Rourke *et al.* 1999; Johnson *et al.* 2003; O'Rourke *et al.* 2004). Additionally, an association was recently demonstrated between susceptibility to bovine spongiform encephalopathy (BSE) and specific insertion/deletion (indel) polymorphisms within the putative *PRNP* promoter and intron 1 for several German cattle breeds (Sander *et al.* 2004), supporting the hypothesis that mutations potentially influencing the level of bovine *PRNP* expression may also influence incubation time and susceptibility to BSE (Bossers *et al.* 1996; Sander *et al.* 2004).

While brucellosis has been effectively eliminated from most bison populations in North America, two substantial reservoirs of brucellosis-infected bison remain in Yellowstone National Park (NP; ID, MT, WY, USA) and Wood Buffalo NP (AB, Canada; Meyer & Meagher 1995; Joly & Messier 2004). Following the initial identification of brucellosis in Yellowstone NP bison (Mohler 1917), multiple studies have documented *Brucella* spp. antibodies (Rush 1932; Tunnicliff & Marsh 1935) as well as *B. abortus* biovars 1 and 2 (Roffe *et al.* 1999; Rhyan *et al.* 2001) in Yellowstone NP bison. Moreover, serological evidence of exposure to *Brucella* spp. has remained relatively high (50–60%) among Yellowstone NP bison for the past 70 years (Tunnicliff & Marsh 1935; Rhyan *et al.* 2001).

Herein, we evaluate the frequencies of bison *PRNP* exon 3 allelic variants and genotypes for six US bison populations (four federal, one private, and one state) and investigate whether variation within exon 3 is significantly associated with *Brucella* spp. antibodies in Yellowstone NP bison. The distribution of *PRNP* exon 3 alleles and genotypes for three bison populations that were either founded from seronegative stock or subjected to test-and-slaughter management to eradicate brucellosis are compared with those observed for Yellowstone NP bison. Additionally, we investigate the frequencies of known bovine *PRNP* indel polymorphisms within the putative bison *PRNP* promoter, intron 1 and 3′-untranslated region (UTR; Hills *et al.* 2001, 2003; Sander *et al.* 2004).

Materials and methods

Bison sampling and serology

Collectively, 198 bison from four US national parks, one private, and one state herd were utilized to investigate the frequencies of *PRNP* allelic variants and genotypes (Table 1). Yellowstone NP samples were obtained non-selectively with regard to pregnancy and/or *Brucella* spp. serology from bison subjected to management actions as they moved to the park boundaries near West Yellowstone, Montana (MT). From these samples, the following selection criteria were used for the current study: approximately equal sex ratio, proportionate sampling from ages $1–10^{+}$ as determined by established aging methods (Fuller 1959; Moffitt 1998), sampling from various collection dates and West Yellowstone NP locals, and approximately equal distributions of *Brucella* spp. serological phenotypes (seropositive/seronegative). Samples from the remaining five herds were selected randomly from previously established repositories.

Brucella spp. antibodies were detected in serum samples from Yellowstone NP bison postmortem from 1997 ($n = 27$) and 1999 ($n = 39$) using the complement fixation, Rivanol precipitation, buffered acidified plate antigen, standard plate agglutination, standard tube agglutination, and card tests (Nielsen 2002). Because the effectiveness of

Table 1 Distribution of bison samples by local and sex.

Bison herd	Abbreviation	Location	Total	Male	Female
Castle Rock[1]	CR	NM	20	5	15
Grand Teton National Park[2]	GT	WY	15	4	11
Henry Mountains[3]	HM	UT	20	7	13
Theodore Roosevelt National Park[4]	TR	ND	10	3	7
Wind Cave National Park[5]	WC	SD	16	3	13
Yellowstone National Park[6]	YNP	ID, MT, WY	117	58	59
Total			198	80	118

[1]Private herd primarily established from seronegative YNP bison (D. Hunter, pers. comm.).
[2]Established from YNP bison in 1948 and supplemented with TR bison in 1964 (NPS 1996); population chronically infected with brucellosis (Meyer & Meagher 1995).
[3]Founded exclusively from YNP bison in 1940s; brucellosis eradicated through test-and-slaughter management in 1960s (Dowling 1990).
[4]South unit; no current or historical evidence of brucellosis (M. Oehler, pers. comm.).
[5]Founded partially from YNP (Coder 1975); brucellosis eradicated through test-and-slaughter management during 1950–1970 (B. Muenchau, pers. comm.).
[6]Samples collected near West Yellowstone, Montana; population chronically infected with brucellosis (Meyer & Meagher 1995).

individual serological tests vary in bison (Williams *et al.* 1997), only samples with consensus serological results were utilized. Fluorescence polarization assay (Gall *et al.* 2000) was used to evaluate the presence of *Brucella* spp. antibodies in samples collected in 2002 ($n = 44$) and 2003 ($n = 7$).

PRNP exon 3 amplification and sequencing

Genomic DNA was isolated by spotting whole blood on Whatman Bioscience FTA® Classic Cards following the recommended protocol (Whatman Inc., Clifton, NJ, USA), by using the SUPER QUICK-GENE DNA Isolation kit (AGTC Inc., Denver, CO, USA) on white blood cell isolates or via pulverization/proteinase K treatment of tissues (Halbert *et al.* 2004). Flanking primers SAF1 and SAF2 (Prusiner *et al.* 1993) were used to PCR amplify and sequence bison *PRNP* exon 3 (Seabury & Derr 2003). Bidirectional sequencing of 96 bison samples from four US federal and one private bison herd was previously employed to evaluate the number of variable sites within bison *PRNP* exon 3 (Seabury *et al.* 2004a), with 71 of those samples included in the present study. Herein, 127 additional samples were sequenced using SAF1 only. *PRNP* exon 3 genotypes and allelic variants were determined using SeqScape version 1.01 (Applied Biosystems, Foster City, CA, USA), and representative exon 3 alleles were validated via cloning and subsequent bidirectional sequencing following Seabury & Derr (2003).

PRNP promoter, intron 1 and 3′-UTR genotyping

Fluorescent dye-labelled primer combinations (Seabury *et al.* 2004b) targeting known indel polymorphisms in the bovine *PRNP* promoter (+23 bp; Sander *et al.* 2004), intron 1 (+12 bp; Hills *et al.* 2001) and 3′-UTR (+14 bp; Hills *et al.* 2003) were utilized to generate genotypic data for bison from Yellowstone NP ($n = 64$; 24 seropositive, 40 seronegative), Wind Cave National Park NP ($n = 20$), Castle Rock ($n = 20$), and Henry Mountains ($n = 20$). PCR reactions and thermal cycling parameters followed Seabury *et al.* (2004b) with the following modifications: 5-μl reaction with primers *PRNP* 47784F, 47803R, 67976F and 68070R with a 52 °C annealing temperature, and a second 5-μl reaction using primers *PRNP* 49686F and 49777R with the cycling profile of Sander *et al.* (2004). Resulting fragments were co-loaded into a single injection for separation on an ABI 3100 (Applied Biosystems) using an internal size standard (Mapmarker LOW; Bioventures, Inc., Murfreesboro, TN, USA).

Statistical analysis

Bison *PRNP* allele frequencies, genic and genotypic differentiation employing both the probability test (Raymond & Rousset 1995) and the G-based test of Goudet *et al.* (1996) as well as Hardy–Weinberg equilibrium were calculated using GENEPOP 3.1d (Raymond & Rousset 1995). *PRNP* exon 3 data for Yellowstone NP bison were subdivided into two groups based on serology (Table 2) and tested for genic and genotypic differentiation. Population differentiation was examined among all bison herds sampled, including Yellowstone NP both with and without subdivision. Because disease cases often differ from controls within individual genotypic classes, we also used a variation of the Z_{max} test to evaluate associations between specific genotypic classes and serological status in Yellowstone NP bison (Lange 2002). $P < 0.05$ was considered statistically significant.

Results

All bison *PRNP* exon 3 alleles examined possessed six octapeptide repeats and were identical in sequence with the exception of one non-synonymous single nucleotide polymorphism (SNP) at nucleotide position 50 (T50C). The T50C SNP is predicted to result in the amino acid replacement M17T. None of the tested populations deviated from Hardy–Weinberg expectations for this SNP. Observed *PRNP* exon 3 allelic and genotypic frequencies for each bison population examined as well as tests of genic and genotypic differentiation are depicted in Table 3. Significant differences in the distributions of *PRNP* exon 3 alleles and genotypes were detected between Yellowstone NP bison and three other bison populations, each either founded from *Brucella* spp. seronegative stock or subjected to test-and-slaughter management (Table 1). Notably, our samples for

Table 2 Distribution of Yellowstone bison samples by sex, *Brucella* spp. serological status and age.

	Age class[1]										
	Total	1	2	3	4	5	6	7	8	9	10+
Yellowstone											
Female seronegative	23	1	1	8	3	3	1	4	1		1
Female seropositive	36	1	1	7	5	7		3	2	1	9
Male seronegative	21	4	1	7	3	1		1		2	2
Male seropositive	37	1	1	16	9	1	1	5		1	2
Sum	117	7	4	38	20	12	2	13	3	4	14

[1]Bison scored in field as 'adult' considered here as 3-year olds

Table 3 Observed *PRNP* exon 3 allelic and genotypic frequencies from six bison populations. The Yellowstone NP population (YNP) is further subdivided by *Brucella* spp. serological phenotypes into seropositive (YNP+) and seronegative (YNP–) classes. Probabilities for population tests of genic and genotypic differentiation are depicted above and below the diagonal, respectively, in the last seven columns ($P < 0.05$ illustrated in bold; all standard errors were <0.01).

	Allele[1]			Genotype[1]			Genic and genotypic differentiation							
Herd	*n*	T	C	TT	TC	CC	YNP +	YNP –	YNP	GT	TR	CR	WC	HM
YNP +	73	0.514	0.486	0.301	0.425	0.274	–	0.05359	NA[2]	0.42297	0.47863	**0.00026**	**0.00583**	**0.01257**
YNP –	44	0.648	0.352	0.409	0.477	0.114	0.06994	–	NA[2]	0.66664	0.79848	0.05440	0.18667	0.30602
YNP	117	0.564	0.436	0.342	0.444	0.214	NA[2]	NA[2]	–	0.84522	0.81513	**0.00208**	**0.02145**	**0.03399**
GT	15	0.600	0.400	0.267	0.667	0.067	0.43923	0.65242	0.85227	–	1.00000	0.05578	0.16801	0.20469
TR	10	0.600	0.400	0.300	0.600	0.100	0.51240	0.78990	0.82476	1.00000	–	0.11272	0.21174	0.25001
CR	20	0.825	0.175	0.700	0.250	0.050	**0.00183**	0.06113	**0.00233**	**0.04905**	0.12022	–	0.76515	0.57814
WC	16	0.781	0.219	0.625	0.313	0.063	**0.01063**	0.18886	**0.03151**	0.14260	0.21646	0.78500	–	0.78844
HM	20	0.750	0.250	0.600	0.300	0.100	**0.01307**	0.31785	**0.04908**	0.19035	0.38893	0.61824	0.80418	–

See Table 1 for herd abbreviations and histories.

[1]Frequencies rounded to three decimal places.

[2]Not an appropriate statistical test.

the Castle Rock, Wind Cave NP and Henry Mountains bison herds all possessed a significantly higher frequency of the T allele than bison sampled from Yellowstone NP (Table 3). No significant genic or genotypic differentiation was detected in comparisons between Yellowstone NP, Grand Teton NP and Theodore Roosevelt NP bison (Table 3).

A significant overabundance (20 of 25; 80%; Z_{max} $P = 0.021$) of *Brucella* spp. seropositive bison were detected within the C/C genotypic class in Yellowstone NP. However, no significant overall genic or genotypic ($P = 0.05359$; 0.06994) differentiation was detected between seropositive and seronegative Yellowstone NP bison (Table 3). No significant sex- and/or age-specific associations were detected for seropositive and/or seronegative Yellowstone NP bison (data not shown), although small sample sizes may have precluded detection (Table 2). Significant differences in the distributions of *PRNP* exon 3 alleles and genotypes were detected between seropositive Yellowstone NP bison and our samples for the Castle Rock, Wind Cave NP and Henry Mountains bison populations (Table 3).

No indel polymorphisms were detected within the targeted regions of the putative bison *PRNP* promoter, intron 1 or 3′-UTR. Based on our fluorescent genotyping assay, all bison were fixed for the following alleles (compared directly with sizes in domestic cattle; Seabury *et al.* 2004b): *PRNP* promoter, 100-bp allele; intron 1, 103-bp allele; 3′-UTR, 97-bp allele.

Discussion

The pattern of nucleotide variation observed for bison *PRNP* exon 3, consisting of one non-synonymous SNP of moderate frequency within the signal sequence, is in marked contrast to that of domestic cattle (Heaton *et al.* 2003; Seabury *et al.* 2004a). Furthermore, while no bison *PRNP* indel variation was detected, exon 3 alleles with four to seven octapeptide repeats (Goldmann *et al.* 1991; Schläpfer *et al.* 1998; Seabury *et al.* 2004a) and indel variation within the promoter, intron 1 and 3′-UTR (Hills *et al.* 2001, 2003; Sander *et al.* 2004) have been documented for domestic cattle. The bison *PRNP* exon 3 T50C non-synonymous SNP and predicted amino acid substitution M17T have not been reported in domestic cattle (Heaton *et al.* 2003; Seabury *et al.* 2004a) or any other species of Bovini (Wopfner *et al.* 1999; Takasuga *et al.* 2003; Seabury *et al.* 2004a). However, the amino acid threonine is widely distributed at the corresponding signal peptide residue for most other mammalian species surveyed (van Rheede *et al.* 2003).

Previous studies have illustrated the sensitivity of PrP^C topology to mutations in the signal sequence and/or transmembrane domain (Hegde *et al.* 1998, 1999; Kim *et al.* 2001; Kim & Hegde 2002), with signal sequence mutations primarily altering the ratio of three topological forms (Hegde *et al.* 1998, 1999) in which PrP^C is synthesized at the endoplasmic reticulum (Kim *et al.* 2001). Furthermore, an association has been suggested between one topological form (^{Ctm}PrP), an uncleaved signal peptide, and neurodegenerative disease (Hegde *et al.* 1998, 1999; Stewart *et al.* 2001; Kim & Hegde 2002). In addition, Lundberg *et al.* (2002) demonstrated that the N-terminal region of mouse PrP^C (residues 1–28), when uncleaved, is a cell-penetrating peptide capable of transporting large hydrophilic cargoes through a cell membrane. Therefore, the predicted amino acid polymorphism M17T may have functional implications in PrP^C biogenesis and/or entry of *Brucella* spp. into bison host cells.

The collective body of evidence presented herein is consistent with a tentative association between the T/C bison *PRNP* exon 3 alleles and *Brucella* spp. serological status (Table 3). The genotypic differentiation noted between seropositive Yellowstone NP bison and the Castle Rock, Wind Cave NP and Henry Mountains bison populations is largely influenced by

high T/T genotype frequencies observed in these populations (Table 3), consistent with a significant overabundance of seropositive Yellowstone NP bison within the C/C genotypic class. Because the Grand Teton NP, Wind Cave NP, Castle Rock, and Henry Mountains populations were all founded either in part or entirely from Yellowstone NP bison (Table 1), it is unlikely that unique genetic backgrounds lead to the observed differences in *PRNP* exon 3 allele and genotype frequencies among populations. However, marked differences do exist in the incidence of brucellosis and previous disease management strategies among these populations (Table 1). It is possible that test-and-slaughter management in both the Wind Cave NP and Henry Mountains bison populations, and the seronegative founder event establishing the Castle Rock population, effectively altered the genetic composition of these herds with respect to genes involved in natural resistance to brucellosis.

To date, a cellular receptor for *Brucella* spp. organisms has not been conclusively identified (Gorvel & Moreno 2002). The association noted herein between bison *PRNP* exon 3 and *Brucella* spp. serological status indirectly supports the findings of Watarai *et al.* (2003), further implicating PrP^C as a potential cellular receptor and/or portal protein for *B. abortus*. However, much remains to be understood about the potential relationship between PrP^C and *B. abortus* infection. The opportunistic sampling of Yellowstone NP bison introduced several uncontrollable factors such as variation in the timing and rate of exposure and use of different serological tests among collection years, perhaps somewhat obscuring the true level of association between *PRNP* exon 3 variation and immune response to *Brucella* spp. organisms. Moreover, while both the susceptibility and immune response to brucellosis infection in bison and domestic cattle have traditionally been assumed equivalent, differences in the pathogenesis of *B. abortus* infection (Rhyan *et al.* 2001) and in the efficacy of vaccination with strains 19 (Davis *et al.* 1991) and RB51 (Olsen *et al.* 2003) are known to exist. Additionally, while other loci are likely involved in the establishment of brucellosis infection in bison and other bovids, future experimental challenge and association studies aimed at evaluating variation within the *PRNP* gene for domestic and wild bovids are necessary to further evaluate the magnitude of the relationship between PrP^C and *B. abortus* infection.

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