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# Comparative PRNP genotyping of U.S. cattle sires for potential association with BSE

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***Abstract***

The recent discovery of significant associations between bovine spongiform encephalopathy (BSE) susceptibility in German cattle and the frequency distributions of insertion/deletion (indel) polymorphisms within the bovine *PRNP* gene prompted an evaluation of 132 commercial U.S. artificial insemination (AI) sires from 39 breeds. Forward primer sequences from published primer sets targeting indels within the putative bovine *PRNP* promoter, intron 1, and the 3′ UTR (untranslated region) were synthesized with unique 5′ fluorescent labels and utilized to develop a rapid multiplexed PCR assay for identifying BSE-associated indels as well as facilitating polymorphism analyses and/or marker-assisted selection. Significant differences ($p < 0.05$ all tests) were detected between the frequencies of bovine *PRNP* promoter alleles for 48 healthy German cattle previously described and 132 commercial U.S. cattle sires. The frequency of the 23-bp promoter allele observed for commercial U.S. cattle sires strongly resembled that recently described for 43 BSE-affected German cattle. No significant difference ($p = 0.051$) was detected between the distributions of promoter genotypes for healthy German cattle and our panel of commercial U.S. cattle sires. Interestingly, significant differences ($p < 0.01$; $p < 0.02$) were also noted between the frequencies and distributions of intron 1 alleles and genotypes, respectively, for BSE-affected German cattle and our panel of U.S. cattle sires. No significant allelic or genotypic differences were detected for the 14-bp 3′ UTR indel for any given comparison between German cattle and commercial U.S. cattle sires.

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Transmissible spongiform encephalopathies (TSEs), or prion diseases, are inevitably fatal neurodegenerative diseases that occur in a variety of mammalian species, including humans as well as domestic and wild animals, and are often characterized by dementia and/or ataxia (Collinge 2001; Prusiner 1998). The pathogenic agents of prion diseases are infectious, protease-resistant proteins which arise through modification of the host-encoded normal cellular prion protein ($PrP^C$). Moreover, prion diseases may occur as genetic, infectious, or sporadic disorders. Additionally, while no definitive consensus regarding the precise function of $PrP^C$ has been reached to date (Aguzzi and Hardt 2003; Collinge 2001), it has been suggested to promote synaptic homeostasis (Collinge et al. 1994), aid in neurite outgrowth and neuronal survival (Chen et al. 2003), and function as a cell-surface receptor for signal transduction (Mouillet–Richard et al. 2000).

Bovine spongiform encephalopathy (BSE), resulting from ingestion of scrapie and/or BSE-infected meat and bone meal, has also been implicated in the development of variant Creutzfeld–Jakob disease (vCJD) in humans via consumption of beef from BSE-affected cattle (Bruce et al. 1997; Scott et al. 1999; Collinge 2001; Asante et al. 2002). To date, nonsynonymous single nucleotide polymorphisms (SNPs) within the human and ovine prion protein gene (*PRNP*) have been notably associated with resistance and/or susceptibility to prion diseases (for review see Belt et al. 1995; Collinge 2001; Baylis et al. 2002), and marker-assisted selection programs

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aimed at enriching the frequency of resistant ovine *PRNP* alleles in Dutch and British sheep have ensued (Schreuder et al. 1997; Arnold et al. 2002). Notably, a novel association was recently documented between BSE susceptibility and specific bovine *PRNP* insertion/deletion (indel) polymorphisms within the putative promoter as well as intron 1 of a few German cattle breeds, supporting the hypothesis that mutations potentially influencing the level of bovine *PRNP* expression might also influence incubation time and susceptibility to BSE (Bossers et al. 1996; Sander et al. 2004).

In this study we investigated the frequencies of BSE-associated *PRNP* indels for a diverse panel of commercial U.S. artificial insemination (AI) sires consisting of 39 distinct breeds and compared them to those recently described for healthy and BSE-affected German cattle breeds (Sander et al. 2004). Additionally, using published primer sequences (Sander et al. 2004) we developed and utilized a PCR protocol incorporating fluorescently labeled primer combinations to produce a multiplexed assay for high-throughput interrogation of bovine *PRNP* indels in the putative promoter, intron 1, and the 3′ untranslated region (UTR; Hills et al. 2001; Hills et al. 2003; Sander et al. 2004).

## Materials and methods

To evaluate the frequencies of bovine *PRNP* indels within the putative promoter, intron 1, and the 3′ UTR, we utilized a DNA panel consisting of 132 AI sires from 39 domestic cattle breeds. The source of DNA was spermatozoa purchased through commercial dealers. Names of breeds and sample sizes ($n$) are as follows: Black Angus (4), Beefalo (1), Beefmaster (5), Belgian Blue (4), Blonde D'Aquitaine (5), Braford (4), Brahman (4), Brahmousin (2), Brangus (5), Braunvieh (5), Brown Swiss (4), Charolais (5), Chianina–Chiangus (5), Corriente (1), Gelbvieh (4), Hereford (3), Holstein (4), Jersey (1), Limousin (3), Maine Anjou (4), Murray Grey (2), Nelore (8), Normande (1), Piedmontese (2), Pinzgauer (1), Red Angus (4), Red Brangus (2), Red Poll (1), Romagnola (2), Salers (3), Santa Gertrudis (4), Scottish Highland (1), Senepol (2), Shorthorn (5), Simbrah (3), Simmental (8), Tarentaise (1), Texas Longhorn (4), Three-way-cross (4), and White Park (1). Six of the Nelore were not AI sires. Care was taken to select unrelated sires.

***Multiplexed PCR assay and validation techniques.*** Forward and reverse primer pairs (Sander et al. 2004) targeting known indel polymorphisms in the bovine *PRNP* putative promoter (23-bp insertion; Sander et al. 2004), intron 1 (12-bp insertion; Hills et al. 2001), and the 3′ UTR (14-bp insertion; Hills et al. 2003) were utilized together in single multiplexed 5-μl volume reactions. Forward primers, respectively, were synthesized with 5′ fluorescent labels as follows: PRNP47784F 5′ NED (Applied Biosystems, Foster City, CA); PRNP49686F 5′ 6-FAM (Sigma-Genosys, The Woodlands, TX); PRNP67976F 5′ HEX (Sigma-Genosys). All multiplexed PCR reactions were carried out on GeneAmp 9700 PCR Systems (Applied Biosystems) and consisted of the following: 50–100 ng DNA, 0.375 units *Taq* polymerase (Promega, Madison, WI), 0.64 μM PRNP47784F-NED, 0.64 μM PRNP477883R, 0.64 μM PRNP49686F-6-FAM, 0.64 μM PRNP49777R, 0.52 μM PRNP67976F-HEX, 0.52 μM PRNP68070R, 3 mM $MgCl_2$ (Promega), 500 μM dNTPs (Promega), 1× MasterAmp™ PCR Enhancer (Epicentre, Madison, WI), and 1.5× Reaction Buffer (Promega). Thermal cycling parameters, as optimized in our laboratory, were as follows: 2 min at 96°C; 4 cycles × 30 sec at 96°C, 30 sec at 58°C (−1°C/cycle), 90 sec at 65°C; 31 cycles × 30 sec at 96°C, 30 sec at 54°C, 90 sec at 65°C; 15 min at 65°C. Multiplexed PCR products were separated and analyzed on an ABI 3100 and/or 310 Genetic Analyzer (Applied Biosystems) and sized relative to an internal size standard (MAPMARKER LOW, Bioventures). For comparison of consistency and validation of the multiplex developed, 10 sires were also genotyped via agarose gel electrophoresis following the methods of Sander et al. (2004).

***Statistical analysis.*** The distributions of *PRNP* alleles between all German cattle (pooled healthy + BSE-affected; Sander et al. 2004) and U.S. cattle sires, healthy German cattle and U.S. cattle sires, and BSE-affected German cattle and U.S. cattle sires were tested for significant differences using Fisher's exact test within the program STAT-SAK (G. E. Dallal, freeware). In addition, tests of genic and genotypic differentiation between the aforementioned groups were carried out using the G-based exact test of Goudet et al. (1996) within the program GENEPOP 3.1d (Raymond and Rousset 1995) using the default parameters. In all cases, $p < 0.05$ was considered statistically significant.

## Results

The multiplexed PCR assay developed for the rapid genotyping of BSE-associated bovine *PRNP* indel polymorphisms in the putative promoter and intron 1, as well as known indel polmorphisms within the 3′ UTR, is illustrated in Fig. 1. No significant differences in the distributions of bovine *PRNP* alleles

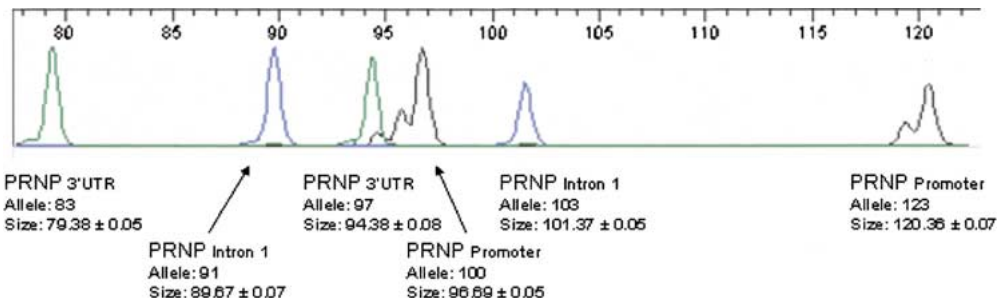


**Fig. 1.** Graphical depiction of the multiplexed PCR products corresponding to indel polymorphisms in the bovine *PRNP* putative promoter, intron 1, and 3′ UTR. Average allele sizes (bp) relative to an internal size standard (MAPMARKER LOW, Bioventures) and the standard deviation are given beneath each of the PCR products generated.

and/or genotypes corresponding to the promoter, intron 1, and 3′ UTR were noted between German cattle as a whole (pooled healthy + BSE-affected) and our panel of U.S. cattle sires. However, significant differences in the allelic and genotypic distributions of bovine *PRNP* indels in the promoter and intron 1 were noted when U.S. cattle sires were compared to healthy as well as BSE-affected German cattle (Table 1; Sander et al. 2004). Specifically, significant differences were detected between the distributions of *PRNP* promoter alleles for healthy German cattle and our panel of U.S. cattle sires (Table 1). Moreover, the frequency of the 23-bp promoter allele observed for our panel of U.S. cattle sires strongly resembled that previously reported for BSE-affected German cattle (Table 1; Sander et al. 2004). No significant difference was detected in the distribution of *PRNP* promoter genotypes between healthy German cattle and our panel of U.S. cattle sires ($p = 0.0510$; Table 1). In contrast, the frequency of the 12-bp intron 1 allele observed for our panel of U.S. cattle sires was identical to that previously reported for healthy German cattle (Table 1; Sander et al. 2004). Significant differences were detected between the distributions of intron 1 alleles and genotypes for BSE-affected German cattle and our panel of U.S. cattle sires (Table 1). No significant differences were detected in the distributions of alleles or genotypes corresponding to the 14-bp indel within the *PRNP* 3′ UTR, and the frequency of the 14-bp allele (+) and corresponding ++ genotype was comparable to that reported for healthy German cattle (Table 1; Sander et al. 2004). Observed *PRNP* indel allele and genotype frequencies, subdivided by domestic breed for those breeds where three or more sires were sampled, are depicted in Table 2.

**Table 1. Allelic and genotypic frequencies observed for bovine *PRNP* putative promoter, intron 1, and 3′ UTR indel polymorphisms**

| | | | *Allele* | | *Genotype* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Locus* | | ***(n)*** | + | − | ++ | +− | −− | *Healthy*[a] | *Affected*[b] | *U.S. sires* |
| Promoter 23-bp indel | Healthy[a] | 48 | 0.43 | 0.57 | 0.21 | 0.44 | 0.35 | — | **0.0296[c]; 0.0316[d]** | **0.0321[c]; 0.0340[d]** |
| | Affected[b] | 43 | 0.27 | 0.73 | 0.05 | 0.44 | 0.51 | **0.0330** | — | 0.5871[c]; 0.5895[d] |
| | U.S. sires | 132 | 0.30 | 0.70 | 0.14 | 0.32 | 0.54 | 0.0510[e] | 0.6141[e] | — |
| Intron 1 12-bp indel | Healthy[a] | 48 | 0.49 | 0.51 | 0.21 | 0.56 | 0.23 | — | **0.0344[c]; 0.0355[d]** | 1.0000[c]; 1.0000[d] |
| | Affected[b] | 43 | 0.33 | 0.67 | 0.09 | 0.47 | 0.44 | **0.0294[e]** | — | **0.0086[c]; 0.0092[d]** |
| | U.S. sires | 132 | 0.49 | 0.51 | 0.32 | 0.35 | 0.33 | 1.0000[e] | **0.0198[e]** | — |
| 3′ UTR[f] 14-bp indel | Healthy[a] | 48 | 0.95 | 0.05 | 0.90 | 0.10 | 0.00 | — | 0.5530[c]; 0.5565[d] | 1.0000[c], 1.0000[d] |
| | Affected[b] | 43 | 0.92 | 0.08 | 0.86 | 0.12 | 0.02 | 0.5773[e] | — | 0.4632[c]; 0.4661[d] |
| | U.S. sires | 132 | 0.94 | 0.06 | 0.89 | 0.11 | 0.00[g] | 0.8137[e] | 0.6366[e] | — |

Differences among healthy and BSE-affected German cattle (Sander et al. 2004) and a panel of commercial U.S. sires were evaluated. Corresponding probabilities are shown in the last 3 columns with genic and genotypic differentiation above and below the diagonal, respectively ($p < 0.05$ depicted in bold).
[a]Healthy German cattle and corresponding data from Sander et al. (2004).
[b]BSE-affected German cattle and corresponding data from Sander et al. (2004).
[c]Probability obtained from Fisher's exact test in STAT-SAK (G. E. Dallal).
[d]Probability obtained from genic differentiation analysis using the G-based exact test of Goudet et al. (1996).
[e]Probability obtained from genotypic differentiation analysis using the G-based exact test of Goudet et al. (1996).
[f]14-bp indel consisting of 1 (−) or 2 (+) repeats.
[g]One sire out of 132 (actual observed frequency = 0.0075).

**Table 2. Observed PRNP indel allele frequencies for cattle breeds where 3 or more sires were sampled**

| | | *Promoter 23-bp indel* | | *Intron 1 12-bp indel* | | *3′ UTR 14-bp indel* | |
|---|---|---|---|---|---|---|---|
| *Domestic cattle breed*[a] | *(n)* | + | − | + | − | + | − |
| Angus[b] | 8 | 0.188 | 0.812 | 0.188 | 0.812 | 1.000 | 0.000 |
| Beefmaster | 5 | 0.000 | 1.000 | 0.200 | 0.800 | 1.000 | 0.000 |
| Belgian Blue | 4 | 0.250 | 0.750 | 0.250 | 0.750 | 1.000 | 0.000 |
| Blonde d′[c] | 5 | 0.500 | 0.500 | 0.600 | 0.400 | 0.900 | 0.100 |
| Braford | 4 | 0.125 | 0.875 | 0.250 | 0.750 | 0.500 | 0.500 |
| Brahman | 4 | 0.250 | 0.750 | 1.000 | 0.000 | 0.875 | 0.125 |
| Brangus[d] | 7 | 0.071 | 0.929 | 0.571 | 0.429 | 1.000 | 0.000 |
| Braunvieh | 5 | 0.400 | 0.600 | 0.700 | 0.300 | 0.900 | 0.100 |
| Brown Swiss | 4 | 1.000 | 0.000 | 1.000 | 0.000 | 1.000 | 0.000 |
| Charolais | 5 | 0.600 | 0.400 | 0.700 | 0.300 | 1.000 | 0.000 |
| Chianina[e] | 5 | 0.500 | 0.500 | 0.500 | 0.500 | 0.900 | 0.100 |
| Gelbvieh | 4 | 0.250 | 0.750 | 0.375 | 0.625 | 1.000 | 0.000 |
| Hereford | 3 | 0.500 | 0.500 | 0.500 | 0.500 | 1.000 | 0.000 |
| Holstein | 4 | 0.375 | 0.625 | 0.375 | 0.625 | 1.000 | 0.000 |
| Limousin | 3 | 0.500 | 0.500 | 0.833 | 0.167 | 1.000 | 0.000 |
| Maine Anjou | 4 | 0.250 | 0.750 | 0.375 | 0.625 | 1.000 | 0.000 |
| Nelore | 8 | 0.000 | 1.000 | 1.000 | 0.000 | 1.000 | 0.000 |
| Salers | 3 | 0.333 | 0.667 | 0.333 | 0.667 | 0.833 | 0.167 |
| Santa Gertrudis | 4 | 0.000 | 1.000 | 0.375 | 0.625 | 1.000 | 0.000 |
| Shorthorn | 5 | 0.100 | 0.900 | 0.100 | 0.900 | 1.000 | 0.000 |
| Simbrah | 3 | 0.333 | 0.667 | 0.833 | 0.167 | 0.833 | 0.167 |
| Simmental | 8 | 0.000 | 1.000 | 0.000 | 1.000 | 0.875 | 0.125 |
| Texas Longhorn | 4 | 0.625 | 0.375 | 0.750 | 0.250 | 1.000 | 0.000 |

[a]Allele frequencies depicted herein are not intended to represent entire breeds.
[b]Black and Red Angus combined.
[c]Blonde d′ Aquitaine.
[d]Black and Red Brangus combined.
[e]Chianina/Chiangus (Chianina × Angus).

## Discussion

Unlike previous bovine *PRNP* studies involving U.S. cattle (Ryan and Womack 1993; Neibergs et al. 1994; Heaton et al. 2003), the study presented and described here provides the first comprehensive survey of bovine *PRNP* indel polymorphisms corresponding to the promoter, intron 1, and 3′ UTR, facilitated through the utilization of a large and diverse panel of commercial U.S. AI sires from 39 domestic breeds.

While *PRNP* polymorphisms associated with resistance to prion diseases have previously been described for humans, sheep, and goats (Belt et al. 1995; Collinge 2001; Billinis et al. 2002), a similar association has only recently been extended to domestic cattle (Sander et al. 2004). Additionally, it should be emphasized that the strength of the association uncovered in German cattle still remains to be evaluated through experimental challenge. Nevertheless, the results of Sander et al. (2004) clearly demonstrate that domestic cattle possess *PRNP* alleles and genotypes significantly associated with susceptibility to BSE. Therefore, an assay capable of the rapid identification of cattle possessing *PRNP* genotypes significantly associated with the phenotypic expression of BSE is potentially invaluable to both the domestic cattle industry as well as human health. Herein we have provided such an assay based on data generated for healthy and BSE-affected German cattle (Fig. 1; Sander et al. 2004). Additionally, we have also provided an initial survey of the allelic and genotypic frequencies corresponding to *PRNP* indels previously associated with BSE for specific domestic cattle breeds (Table 2). However, while care was taken to select unrelated sires from each respective breed, caution is necessary when interpreting the relationship between the observed frequencies depicted in Table 2 and potential susceptibility and/or resistance to BSE. Future studies utilizing larger sample sizes are necessary to comprehensively evaluate breed-specific frequencies of *PRNP* indel polymorphisms within the promoter, intron 1, and 3′ UTR for commercial U.S. cattle breeds.

The origin of the significant differences detected between *PRNP* allele and/or genotype distributions for our panel of U.S. cattle sires and German cattle is currently unclear (Table 1). Given that the newly described 23-bp bovine *PRNP* promoter indel

exhibited the most significant association with BSE in German cattle, Sander et al. (2004) subsequently attributed the statistical significance noted between the 12-bp intron 1 indel and BSE to tight linkage between the promoter and intron 1. However, the potential effects and/or role(s) of each polymorphism with respect to bovine *PRNP* expression and BSE susceptibility still remain to be completely elucidated. Furthermore, it should be noted that the 12-bp intron 1 indel was previously determined to lie within a region important for retention of full *PRNP* promoter activity in cultured bovine CKT-1 cells, and the 12-bp deletion was determined to remove a putative binding site for the transcription factor SP1 (Inoue et al. 1997; Hills et al. 2001). Interestingly, our panel of U.S. cattle sires possesses a significantly higher frequency of the 12-bp (+) intron 1 allele and corresponding ++ genotype than BSE-affected German cattle (Table 1), but differs significantly from healthy German cattle with respect to the frequency of the 23-bp (+) promoter allele which previously exhibited the most significant association with BSE status. If our analysis of these data is interpreted with strict adherence to the results of Sander et al. (2004), it appears that our panel of U.S. cattle sires, collectively, are largely susceptible to BSE based on polymorphism data generated for the bovine *PRNP* promoter. However, additional studies focusing on the regulatory effects of *PRNP* indels in the promoter as well as intron 1 are necessary to fully evaluate the implications of the significantly different distributions of BSE-associated *PRNP* alleles and/or genotypes between commercial U.S. cattle sires and German cattle.

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