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Sequence Variation of Bovine Prion Protein Gene in Japanese Cattle (Holstein and Japanese Black)

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ABSTRACT. To assess relationships between nucleotide polymorphisms of the prion protein (*PRNP*) gene and susceptibility to bovine spongiform encephalopathy (BSE), we investigated polymorphisms in the open reading frame (ORF) and 2 upper regions of the *PRNP* gene from 2 Japanese cattle breeds: 863 healthy Holstein cattle, 6 BSE-affected Holstein cattle, and 186 healthy Japanese Black (JB) cattle. In the ORF, we found single-nucleotide polymorphisms (SNPs) at nucleotide positions 234 and 576 and found 5 or 6 copies of the octapeptide repeat, but we did not find any amino acid substitutions. In the upper region, we examined 2 sites of insertion/deletion (indel) polymorphisms: a 23-bp indel in the upper region of exon 1, and a 12-bp indel in the putative promoter region of intron 1. A previous report suggests that the 23-bp indel polymorphism is associated with susceptibility to BSE, but we did not find a difference in allele frequency between healthy and BSE-affected Holstein cattle. There were differences in allele frequency between healthy Holstein and JB cattle at the 23- and 12-bp indels and at the SNPs at nucleotide positions 234 and 576, but there was no difference in allele frequency of the octapeptide repeat. We identified a unique *PRNP* gene lacking a 288-bp segment (96 amino acids) in DNA samples stocked in our laboratory, but this deletion was not found in any of the 1049 cattle examined in the present study. The present results provide data about variations and distribution of the bovine *PRNP* gene.
KEY WORDS: BSE, cattle, polymorphism, prion, *PRNP*.

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Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), Kuru and fatal familial insomnia (FFI) in humans, scrapie in sheep and goats, chronic wasting disease (CWD) in deer and elk, feline spongiform encephalopathy (FSE) in cats, transmissible mink encephalopathy (TME) in minks, and bovine spongiform encephalopathy (BSE) in cattle [26,27]. The hallmarks of TSE are neuronal vacuolation, astrocytosis, and accumulation of a pathogenic, abnormal and protease-resistant isoform of prion protein (PrP), designated PrP^{Sc} or prion, in the central nervous system. PrP^{Sc} is generated from the endogenous cellular prion protein PrP^{C}, encoded by the prion protein gene (*PRNP*), by post-translational modification leading to conformational changes [26, 27]. It has been hypothesized that PrP^{C} plays a role in copper metabolism [20, 32], but the normal functions of PrP^{C} in cells are unclear.

It is generally believed that BSE epidemics in cattle are caused by ingestion of meat and bone meal contaminated with PrP^{Sc} [35]. BSE appears to pose a threat not only to cattle but also to human public health, because the human disease variant CJD (vCJD) is thought to be caused by ingestion of meat or meat products contaminated with BSE [3, 30]. Genetic resistance to TSE is thought to be an important factor in prevention of disease recurrence. In the open reading frame (ORF) of the *PRNP* gene, amino acid polymorphisms associated with prion disease susceptibility and incubation period have been found in humans [24], sheep [2, 4, 13] and mice [34]. Sheep with valine at codon 136 and glutamine at codon 171 ($V^{136}Q^{171}$ animals) exhibit high susceptibility and short incubation period for scrapie, whereas sheep with alanine at codon 136 and arginine at codon 171 ($A^{136}R^{171}$ animals) exhibit resistance to scrapie [2, 13]. An analysis of the ORF of the *PRNP* gene of cattle (*Bos Taurus*) revealed several nucleotide substitutions including an octapeptide repeat polymorphism, but it is unclear whether the polymorphisms correlate with BSE [6, 12,14, 23, 28].

In Japan, the first BSE-affected animal was found in September 2001, and a total of 20 BSE-infected cattle have been found at end of July, 2005. There have been few reports about sequence variations in the *PRNP* gene of Holstein and Japanese Black (JB) cattle, which are widely raised in Japan [31]. The purpose of the present study was to examine variations of the *PRNP* gene in Holstein and JB cattle in Japan, including estimation of the relationship between bovine *PRNP* polymorphisms and occurrence of BSE.

MATERIALS AND METHODS

Animals and DNA samples: We examined 1049 healthy cattle: 863 Holstein cattle from Hokkaido Prefecture in Japan (489 cattle from 8 dairy farms, and 374 cattle from a

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slaughterhouse), and 189 JB cattle from 5 districts in Gifu Prefecture. We also examined 6 BSE-affected Holstein cattle, from Hokkaido (3 cases), Gunma (1 case), Kanagawa (1 case) and Wakayama (1 case) Prefectures (http://www.mhlw.go.jp/topics/0103/tp0308-1.html). DNA was isolated from blood samples from healthy Holstein and JB cattle, and from meat or the medulla oblongata of BSE-affected cattle, using the QIAamp DNA Blood Mini Kit (Qiagen Science, Germantown, MD). Blood was also directly used for polymerase chain reaction (PCR) with an Ampdirect kit (Shimadzu biotech, Kyoto, Japan). The 52 DNA samples (15 samples from sporadic bovine leucosis, 6 samples from enzootic bovine leucosis, 5 samples from tumor, 16 samples from the other diseases and 10 samples from healthy cattle) stocked in our laboratory were examined in the present study.

DNA amplification: We examined 3 regions for nucleotide polymorphisms: a 23-bp indel polymorphism about 1.5 kb upstream of exon 1, a 12-bp indel polymorphism in intron 1, and the entire ORF of exon 3. To determine DNA sequences, 795 bp of the ORF of the bovine *PRNP* gene (nt 65579 to 66373 in accession number AJ298878 [9]) were amplified using the primers BPrP3 (5'GCAGATATAAGTCATCATGGTG) and BPrP4 (5'GAAGATAATGAAAACAGGAAGG), as described by Gombojav *et al.* [7] (Fig. 1). To assay the octapeptide (PHGGGWGQ) repeat and the 288-bp deletion in the ORF of the bovine *PRNP* gene, we directly amplified the N-terminal region (577 to 565 bp) of the ORF using 0.7 μl of blood (863 Holstein and 186 JB cattle) in 25 μl of Ampdirect buffer containing 1 unit of Taq polymerase (Sigma Aldrich, St. Louis, MO), 10 pmol of the primers BPrP3 and SP4 (5'CATTGGTCTTGTTGAAACAC) and 2 mM dNTPs, according to the Ampdirect kit instructions. To examine the 23-bp insertion/deletion (indel) polymorphism in the upper region of exon 1 (A, 23-bp indel in Fig. 1), we amplified 123 or 100 bp using the primers PRNP47784 and PRNP47883R and DNA specimens from 278 Holstein and 186 JB cattle [28]. For the 12-bp indel polymorphism in intron 1 of the bovine *PRNP* gene (B, 12-bp indel in Fig. 1), we amplified 414 or 426 bp using the primers BPrP30 (5'CTTCTCTCTCGCAGAAGCAG) and BPrP32 (5'CCCTTGTTCTTCTGAGCTCC) and DNA specimens from 290 Holstein and 186 JB cattle. The thermal cycling sequence of the amplification was as follows: initial denaturation at 94°C for 10 min; 50 cycles of denaturation at 94°C for 30 s, annealing at the annealing temperature of the specific primer pair for 30 s, and extension at 72°C for 1 min; final extension at 72°C for 7 min. All PCR products were electrophoresed to assess yield and purity on 1.5% to 3.0% agarose gels, and were then photographed. The PCR product for the 12-bp indel (414 or 426 bp) was digested with *Sac*II enzyme and separated on 2% agarose gel to assay for the presence of the 12-bp indel, because the 12-bp indel sequence contains a *Sac*II recognition site.

DNA sequencing: PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) for DNA sequencing. Purified PCR products of the bovine *PRNP* gene were directly sequenced using internal sequencing primers SP1 (5'TTGGTGGCTACATGCTGGGAAG) and SP4. Sequencing was performed using an ABI 310 DNA sequencer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. Alignment of sequences was performed using GENETYX-MAC software (Software Development Co., Tokyo, Japan).

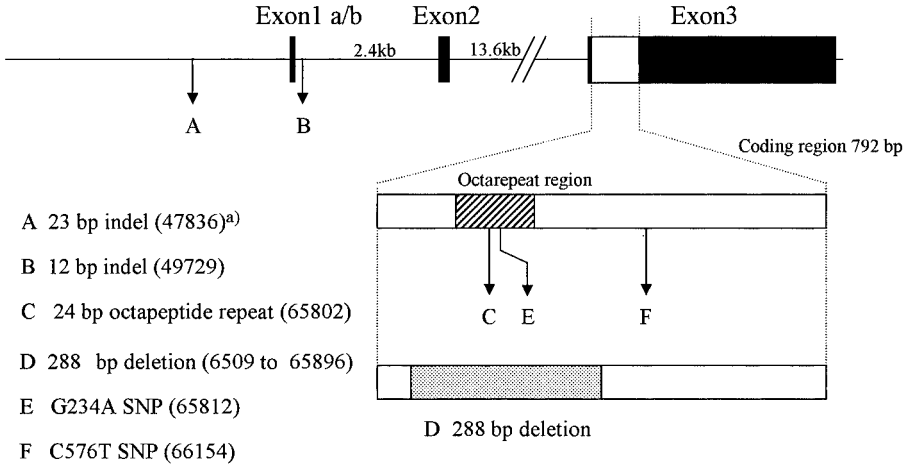

Fig. 1. Diagram of genomic structure of the bovine *PRNP* gene. The 6 polymorphisms (3 indels, 1 deletion and 2 SNPs) found in the present study are indicated by arrows labeled with letters. The 3 *PRNP* exons are represented by black boxes, and the protein-coding region in exon 3 is shown as a white area in the black box [11]. The hatched portion of the coding region indicates the octarepeat region. The dotted portion of the coding region indicates the 96-amino-acid (288-bp) deletion from nt 65609 to 65896 in reference sequence AJ298878 [9]. a) The numbers in parentheses indicate positions in reference sequence AJ298878.

Statistical analysis: We used the χ-square test to assess the significance of associations between allele distribution and BSE among Holstein and JB cattle.

RESULTS

Polymorphisms in the upstream region: Representative results for the 23-bp indel are shown in Fig. 2A. Cattle homozygous for the 23-bp deletion or insertion showed one distinct band, at 100 or 123 bp, respectively (Fig. 2A, designated by –/– and +/+). Cattle heterozygous for the 23-bp indel showed two bands: at 123 and 100 bp (Fig. 2A, +/–). The majority of the Holstein cattle tested for the 23-bp indel (62%) were homozygous for the 23-bp deletion, whereas the majority of the JB cattle (61%) were heterozygous for the 23-bp indel (Table 1). For cattle homozygous for the 12-bp insertion, the *Sac*II enzyme cleaved the PCR product (426 bp) into two fragments: 276 and 150 bp. Cattle homozygous for the 12-bp deletion showed a single band at 414 bp. Cattle heterozygous for the 12-bp indel showed three distinct bands: 414, 276 and 150 bp (Fig. 2B, +/–). Most of the Holstein cattle tested for the 12-bp indel were either heterozygous or were homozygous for the 12-bp deletion, but 61% of the JB cattle were heterozygous.

Among the 6 BSE-affected cattle, 2 cattle were heterozygous at both the 23- and 12-bp indels, and 4 cattle were homozygous at both the 23- and 12-bp indels.

Polymorphisms of ORF in PRNP: In the present study, we found a mutant with a 288-bp deletion (96 amino acids from codon 11 to codon 108) among 52 DNA samples stocked in our laboratory (Fig. 1). The N-terminal region amplified from the 288-bp-deletion mutant showed two bands: one band containing 6 copies of the octapeptide repeat (575 bp), and the other small band (287 bp) with the 288-bp deletion (Fig 2D). None of the 1049 cattle tested carried the 288-bp deletion in the *PRNP* gene. Among both Holstein and JB cattle, the predomininat genotype of the octapeptide repeat was homozygosity for the 6-copy allele (575 bp in Fig. 2C, designated as 6/6), followed by heterozygosity for 5 and 6 copies (Fig. 2C, 6/5) (Table 1). Only 4 Holstein cattle and none of the JB cattle were homozygous for 5 copies of the octapeptide repeat (Fig. 2C, 5/5). All BSE-affected cattle had 6 copies of the octapeptide repeat. We detected 2 single-nucleotide polymorphisms (SNPs) at G234A and C576T in 232 Holstein and 186 JB cattle (Table 1). At the C576T SNP, none of the Holstein cattle were homozygous for thymine, but 28 of the JB cattle were homozygous for thymine.

Statistical analysis of nucleotide polymorphisms: Allele frequencies of nucleotide polymorphisms at the 2 indels (23 and 12 bp), the octapeptide repeat and 2 SNPs (nt 234 and 576) are shown in Table 2. There was no significant difference between healthy and BSE-affected Holstein cattle at any of the nucleotide polymorphisms. At the 23- and 12-bp indels and the G234A and C576T SNPs, there were significant differences in allele frequency between healthy Holstein and JB cattle (Table 2).

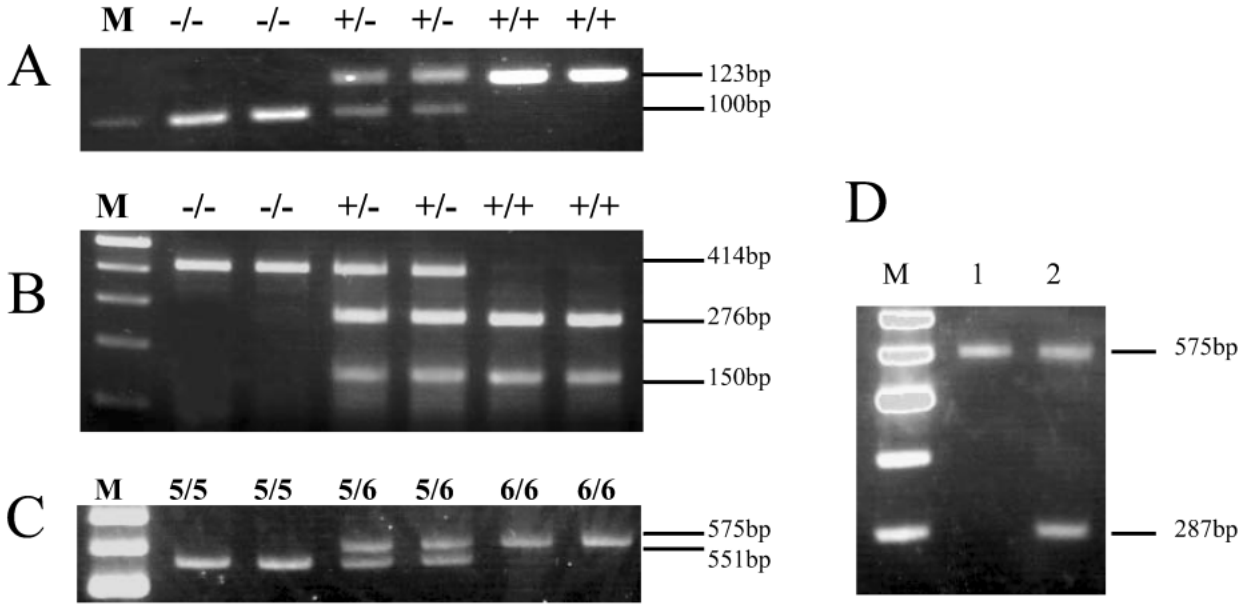

Fig. 2. Genotyping of insertion or deletion polymorphisms by agarose gel electrophoresis. A) Genotypes of 6 different cattle are indicated as presence (+) or absence (–) of 23-bp insertion. B) Genotypes of *Sac*II-digested PCR products from 6 different cattle are indicated as presence (+) or absence (–) of 12-bp insertion. C) Genotypes of octapeptide repeats (homozygote 5/5, heterozygote 5/6 and homozygote 6/6). D) Genotypes of N-terminal region of *PRNP* gene amplified using primers BPrP3 and SP4: lane 1, PCR product from 6/6 homozygote; lane 2, PCR product from 288-bp-deletion mutant. M, 100-bp DNA size marker.

Table 1. Genotype frequency of polymorphisms among healthy Holstein, healthy JB cattle and BSE-affected Holstein cattle

Breed and location		N[f]	23-bp indel[a]			12-bp indel[b]			Octarepeat[c]			G234A[d]			C576T[e]		
			+/+	+/–	–/–	+/+	+/–	–/–	6/6	6/5	5/5	G/G	G/A	A/A	C/C	C/T	T/T
Healthy Holstein	A	15	1	8	6	2	8	5	13	2	0	6	7	2	15	0	0
	B	27	3	12	11	4	16	6	23	4	0	17	7	3	27	0	0
	C	168	0	36	127	0	45	117	148	19	1	59	11	4	72	2	0
	D	74	10	34	30	11	37	26	61	13	0	–	–	–	–	–	–
	E	13	–	–	–	4	4	5	10	3	0	7	2	4	13	0	0
	F	51	–	–	–	–	–	–	47	4	0	32	10	9	51	0	0
	G	125	–	–	–	–	–	–	109	14	2	43	9	0	52	0	0
	H	16	–	–	–	–	–	–	14	2	0	–	–	–	–	–	–
	I	374	–	–	–	–	–	–	340	33	1	–	–	–	–	–	–
Total healthy Holstein		863	14	90	174	21	110	159	765	94	4	164	46	22	230	2	0
Healthy JB[g]																	
	J	37	3	29	5	3	26	8	37	0	0	6	25	6	13	20	4
	K	37	7	24	6	10	22	5	30	7	0	11	19	7	18	14	5
	L	31	0	11	20	0	13	18	28	3	0	16	15	0	4	16	11
	M	40	4	22	14	6	24	10	37	3	0	13	21	6	17	20	3
	N	41	5	28	8	5	28	8	41	0	0	10	27	4	15	21	5
Total healthy JB		186	19	114	53	24	113	49	173	13	0	56	107	23	67	91	28
Total healthy Holstein and healthy JB		1049	33	204	227	45	223	208	938	107	4	220	153	45	297	93	28
BSE-affected Holstein		6	0	2	4	0	2	4	6	0	0	4	1	1	6	0	0

a) The 23-bp indel polymorphism in the upper region of exon 1 in Fig.1.
b) The 12-bp indel polymorphism in intron 1 in Fig.1.
c) Octarepeat polymorphism of the coding region.
d),e) Polymorphism at nt 234 and 576 in the ORF, respectively.
f) Number of samples collected.
g) JB, Japanese Black.

Table 2. Allele frequency of nucleotide polymorphisms among healthy Holstein, healthy JB cattle and BSE-affected Holstein cattle

Breed	Nucleotide polymorphisms																	
	23-bp insertion				12-bp insertion				Octarepeat[e]				G234A				C576T			
	n[a]	+[b]	–[c]	*p*[d]	n	+	–	*p*	n	6	5	*p*	n	G	A	*p*	n	C	T	*p*
Healthy Holstein	278	0.21	0.79		290	0.26	0.74		863	0.94	0.06		232	0.81	0.19		232	1.00	0	
BSE Holstein	6	0.17	0.83	n.s[f]	6	0.17	0.83	n.s	6	1.00	0	n.s	6	0.75	0.25	n.s	6	1.00	0	n.s
Subtotal	284	0.21	0.79		296	0.27	0.74		869	0.94	0.06		238	0.80	0.20		238	1.00	0	
Healthy JB	186	0.41	0.59	<0.01	186	0.43	0.57	<0.01	186	0.97	0.03	n.s	186	0.56	0.41	<0.01	186	0.60	0.40	<0.01
Healthy JB and Holstein	464	0.29	0.71		476	0.33	0.67		1049	0.95	0.05		418	0.71	0.29		418	0.82	0.18	

a) n, number of cattle examined.
b) +, 23-bp or 12-bp insertion.
c) –, 23-bp or 12-bp deletion.
d) $p<0.01$, significant difference among the compared values.
e) Five or 6 copies of octapeptide repeat.
f) n.s, not significant.

DISCUSSION

PRNP polymorphisms are associated with variation in susceptibility to prion disease in humans [24], sheep [2, 4, 5, 13] and mice [34]. There have been many studies of relationships between *PRNP* polymorphisms and susceptibility to BSE in cattle [8, 12, 14, 22, 25, 28, 29, 31, 33]. However, few studies have found significant relationships between *PRNP* polymorphisms and occurrence of BSE [28]. The number of BSE-affected animals tested in the present study (6 cattle) was too small for reliable estimation of genetic relationships between bovine *PRNP* polymorphisms and occurrence of BSE, and we found no evidence of such relationships.

In the present study, we assayed for *PRNP* polymorphisms in 863 healthy Holstein cattle and 186 healthy JB cattle. The only polymorphism caused amino acid substitutions in the ORF was the octapeptide repeat. We observed only 2 SNPs, at G234A and C576T; these SNPs have frequently been observed in other studies [8, 10, 13, 17, 28, 31]. Only 2 of the 863 Holstein cattle were heterozygous for the C576T polymorphism, whereas 91 of the 186 JB cattle were heterozygous at C576T (Table 2). Similar differences in allele frequencies between the 2 cattle breeds were found at the 23- and 12-bp indels and the G234A SNP. These differences may be due to inherent properties of the cattle breeds or differences in the breeding systems used for Holstein and JB cattle. The allele frequencies of the bovine *PRNP* gene observed in the present study for JB cattle suggest a breeding system in which a limited number of bulls is used. Takasuga *et al.* [31] found 13 SNPs (including 2 amino acid substitutions) in indigenous Indonesian cattle, which carry more mutations than Holstein and JB cattle. It is generally thought that artificial insemination, which is widely used to breed dairy and beef cattle, decreases genetic variation and produces uniform genetic properties at the DNA level. Holstein and JB cattle raised in Japan appear to have fewer mutation sites in the *PRNP* gene than indigenous breeds such as indigenous Indonesian cattle [31].

Several cattle breeds have been shown to have octarepeat polymorphisms in the ORF of the *PRNP* gene [5, 22, 25, 33], and 3 alleles of the octapeptide repeat (5, 6 and 7 copies) have been reported. In the present study, none of the cattle had 7 copies of the octapeptide repeat, and the frequency of the 5-copy allele was very low (Tables 1 and 2). The predominant genotype of the octapeptide repeat in the present Holstein and JB cattle was homozygosity of the 6-copy allele. Variability of the octapeptide repeat has not been found to correlate with incidence of BSE in cattle [5, 14, 23], and no BSE-affected cattle have been found to be homozygous for the 5-copy allele. We found no significant differences in the genotype distribution of the SNPs or octarepeat polymorphisms between healthy and BSE-affected Holstein cattle (Table 2).

In the present study, we investigated DNA polymorphisms in 2 regions upstream from the ORF: a 23-bp indel in the upper region of exon 1, and a 12-bp indel in intron 1 (Fig. 1). The available evidence suggests that polymorphisms in these regions affect transcription of the *PRNP* gene [15, 18, 21]. In a previous study, the 23-bp insertion was found to occur more frequently in healthy cattle than BSE-affected cattle [28]. Among Holstein cattle in Japan, the 23-bp insertion has been found to have a lower allele frequency than the 23-bp deletion. We speculated that polymorphism of the 12-bp indel might affect expression levels of the *PRNP* gene, because the indel is in the promoter region of intron 1 and contains a putative Sp1-binding consensus sequence [9, 15, 18, 19]. It has been reported that a GC-rich region and Sp1-binding sequence upstream of exon 1 are both important factors in PRNP transcription [1, 15, 18], but the effects of this Sp1 sequence in intron 1 are unclear. Further research is needed to clarify the effects of the12-bp indel on expression of the *PRNP* gene.

In the present study, we identified a *PRNP* gene mutant with a 288-bp deletion in the ORF (Figs. 1 and 2D), in a specimen from our DNA stock samples. A prion protein with such an internal deletion may confer resistance to prion disease infection or act as a dominant-negative mutant that inhibits prion propagation in the cell [17, 36]. Accurate determination of the distribution of this deletion could help clarify whether it has a preventive effect against prion disease. However, we did not detect this 288-bp deletion in the *PRNP* gene of any of the present 1049 cattle. Our stock DNA specimen with the 288-bp deletion in the *PRNP* gene was obtained from a calf (C928) with the calf form of sporadic bovine leucosis (SBL) [16]. SBL appears to be caused by a somatic mutation in immature pre-B cells, and it is unclear whether the malignant process of SBL is related to an internal deletion in the *PRNP* gene. If the internal deletion in the *PRNP* gene found in specimen C928 is caused by a somatic mutation that is related to the malignant transformation of SBL, it is unlikely that such a deletion would be found in healthy cattle. We examined the bovine *PRNP* gene from the other 7 specimens of SBL calves, but none of those specimens had the same deletion (data not shown).

In conclusion, we found an extremely small number of *PRNP* polymorphic sites in the 2 cattle breeds examined in the present study, and we found no association between these polymorphisms and BSE. Because the number of BSE-affected animals tested was small, further genetic investigations using many samples from BSE cattle can be useful for assessment of the risk of BSE in Japan.

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