Evaluation of *PRNP* Expression Based on Genotypes and Alleles of Two Indel Loci in the Medulla Oblongata of Japanese Black and Japanese Brown Cattle

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Abstract

**Background:** Prion protein (PrP) level plays the central role in bovine spongiform encephalopathy (BSE) susceptibility. Increasing the level of PrP decreases incubation period for this disease. Therefore, studying the expression of the cellular PrP or at least the messenger RNA might be used in selection for preventing the propagation of BSE and other prion diseases. Two insertion/deletion (indel) variations have been tentatively associated with susceptibility/resistance of cattle to classical BSE.

**Methodology/Principal Findings:** We studied the expression of each genotype at the two indel sites in Japanese Black (JB) and Japanese Brown (JBr) cattle breeds by a standard curve method of real-time PCR. Five diplotype subdivide into two categories were selected from each breed. The two cattle breeds were considered differently. Expression of *PRNP* was significantly (*p*<0.0001) greater in the homozygous deletion genotype at the 23-bp locus in JB breed. Compared to the homozygous genotypes, the expression of *PRNP* was significantly greater in the heterozygous genotype at the 12-bp locus in JB (*p*<0.0001) and in JBr (*p*= 0.0394) breeds. In addition, there was a statistical significance in the *PRNP* levels between the insertion and the deletion alleles of the 23-bp locus in JB (*p* = 0.0033) as well as in JBr (*p* = 0.0032). There was no significance in relation to sex, age, geographical location or due to their interactions (*p*>0.05).

**Conclusion:** Our results suggest that the del/del genotype or at least its del allele may modulate the expression of *PRNP* at the 23-bp locus in the medulla oblongata of these cattle breeds.

Introduction

Genetic variations in the prion protein gene (*PRNP*) are linked to the occurrence of transmissible spongiform encephalopathies (TSEs) also called prion diseases in humans, sheep and mice [1–4]. TSEs are characterized by abnormal deposits of a protease-resistant isoform of the host genome encoded prion protein (PrP) and are unique in that they can manifest through acquired, inherited, or sporadic origins [5]. Polymorphisms in two regulatory regions in the bovine *PRNP* have been tentatively associated with classical BSE (cBSE) incidences in a few cattle populations [6–8]. A 23-bp insertion/deletion (indel) polymorphism in the promoter contains a binding site for the repressor protein 58 (RP58) and a 12-bp indel in intron 1 has a binding site for the transcription factor specificity protein 1 (SP1). The presence or absence of these binding sites modulate the expression of *PRNP* and possibly the expression of PrP in species. Expression of the cellular PrP is necessary for the transmission and propagation of prion diseases [9]. Based on reporter gene assays, increased level of PrP decreases the incubation period for cBSE [10].

BSE cases in Japan rose to 36 in 2010. Of these, 33 cases were reported in Holstein-Friesian (HF) animals while three were reported in local Japanese Black (JB) cattle. So far a number of Japanese cattle populations have been analyzed for various polymorphisms in the *PRNP*. Populations analyzed include the JB breed [11], Japanese Brown (JBr) breed and other local breeds [12,13]. The aim of the present study was to examine the effect of genotypes of the 23-bp and 12-bp indel loci in the expression of *PRNP* in JB and JBr breeds.

Results

DNA genotyping and diplotype analysis

A total of 218 animals including 120 JB and 98 JBr were genotyped for polymorphisms in the 23-bp and 12-bp indel loci of the *PRNP*. Both 23-bp and 12-bp loci were polymorphic in both breeds. At the 23-bp locus, frequency of the deletion homozygous
greatest diplotype (29.82%). Five diplotypes namely
breeds. Considering the breeds together, the
diplotypes observed in 20.00% of the JB animals and 5.10% of the JBr
animals. The diplotype observed in 16.33% of the JBr animals and in 5.00% of the JB
animals. The diplotype observed in 10.00% of the JB animals and 7.14% of the JBr animals was the −−/+ was
observed in 20.00% of the JB animals and 5.10% of the JBr
animals. The −−/+ diplotype was observed in 10.00% of the JB
animals. The −−/+ diplotype was observed in the JB breed only. Diplotypes ++/−, ++/−, −−/+ were rare in both
breeds. Considering the breeds together, the −−/+ was the
greatest diplotype (29.82%). Five diplotypes namely ++/++, +−/+,
−−++ −−−−−− were selected for gene expression analyses in the JB breed and five others (++/−+, 
+−/−+, −−/−+, −−++ +−/+−) were selected for the same purpose in the JBr breed. Gene expression was evaluated among
these diplotypes to achieve the study objectives.

**PRNP expression based on genotypes of the 23-bp promoter indel**

To evaluate the expression of PRNP among genotypes of the 23-bp promoter indel, three diplotypes were selected from each breed. Diplootypes ++/++ (n = 6), +−/+ (n = 8), and −−/− (n = 5) were selected in the JB breed whereas diplotypes ++/− (n = 3), 
+−/+ (n = 8) and −−/− (n = 5) were selected in the JBr
breed. The left side of these diplotypes constitute of genotypes ++,
+− and −− of the 23-bp locus whereas the right which represent
the 12-bp locus has a similar genotypes in all diplotypes. It was
presumed that a similar genotype at the 12-bp locus would have
similar effect in these diplotypes and therefore the gene expression
was compared among genotypes of the 23-bp locus on the left side.
The 12-bp locus was represented by the ++ genotype in the JB
breed and +− in the JBr breed. The expression of PRNP relative to

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**Table 1. Genotype and gene frequencies of PRNP polymorphisms in JB and JBr breeds.**

| PRNP site     | Breed | n  | genotype frequency | allele frequency |
|---------------|-------|----|--------------------|------------------|
|               |       |    | ++/++              | ++/−            |
| Prmoter 23-bp indel | JB    | 120| 0.08 0.30 0.62     | 0.23 0.77        |
|               | JBr   | 98 | 0.19 0.50 0.31     | 0.44 0.56        |
| Intron 12-bp indel | JB    | 120| 0.23 0.38 0.39     | 0.42 0.58        |
|               | JBr   | 98 | 0.23 0.47 0.30     | 0.47 0.53        |

n: sample size; JB: Japanese Black cattle; JBr: Japanese Brown cattle.
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**Table 2. Diplople frequencies for two PRNP indel polymorphisms in JB and JBr breeds.**

| Diplople  | JB n = 120 (%) | JBr n = 98 (%) | Total n = 218 (%) |
|-----------|---------------|---------------|------------------|
| ++/++     | 6 (5.00)      | 16 (16.33)    | 22 (10.09)       |
| ++/−      | 0 (0.00)      | 3 (3.06)      | 3 (1.38)         |
| +−/−      | 4 (3.33)      | 0 (0.00)      | 4 (1.83)         |
| +−/+      | 12 (10.00)    | 7 (7.14)      | 19 (8.72)        |
| +−/−      | 21 (17.50)    | 38 (38.78)    | 59 (27.06)       |
| +−/−      | 3 (2.50)      | 4 (4.08)      | 7 (3.21)         |
| −−/−      | 10 (8.33)     | 0 (0.00)      | 10 (4.59)        |
| −−/+      | 24 (20.00)    | 5 (5.10)      | 29 (13.30)       |
| −−/−      | 40 (33.33)    | 25 (25.51)    | 65 (29.82)       |

n: sample size; (%): percentage of a diplotype; JB: Japanese Black cattle; JBr: Japanese Brown cattle.
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**Table 3. PRNP expression for various diplotypes in JB and JBr breeds.**

| Breed | Diplople  | n   | Mean PRNP | SD  | SE  |
|-------|-----------|-----|-----------|-----|-----|
| JB    | ++/++     | 6   | 0.2721    | 0.067 | 0.033 |
|       | +−/−      | 8   | 0.3017    | 0.119 | 0.042 |
|       | −−/++     | 8   | 0.3314    | 0.076 | 0.027 |
|       | −−/−      | 10  | 0.3971    | 0.134 | 0.043 |
|       | −−/−      | 10  | 0.2552    | 0.034 | 0.011 |
|       | +−/+      | 20  | 0.0492    | 0.013 | 0.003 |
|       | +−/−      | 20  | 0.0899    | 0.039 | 0.009 |
| JBr   | ++/++     | 3   | 0.0801    | 0.012 | 0.007 |
|       | +−/−      | 6   | 0.0838    | 0.016 | 0.007 |
|       | −−/−      | 5   | 0.1076    | 0.049 | 0.022 |
|       | +−/+      | 6   | 0.1212    | 0.010 | 0.004 |
|       | +−/−      | 12  | 0.1301    | 0.052 | 0.010 |
|       | −−/−      | 4   | 0.0984    | 0.019 | 0.009 |
|       | +−/+      | 8   | 0.1419    | 0.018 | 0.006 |
|       | +−/−      | 7   | 0.1787    | 0.040 | 0.015 |

n: sample size; PRNP: PRNP/ACTB ratio; SD: Standard deviation; SE: Standard error; JB: Japanese Black cattle; JBr: Japanese Brown cattle.
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The Effect of Indel Genotypes in PRNP Expression
PRNP levels than those with the ++ or the ++ at the 23-bp locus in both breeds (Figure 1). The expression of PRNP was significantly different (p = 0.0001) among genotypes of the 23-bp locus in JB breed but there was no statistical significance (p>0.05) among these genotypes in the JBr breed. Also, there was no significance in relation to sex, age, regions or farms of the animals and their interactions (p>0.05).

**PRNP expression based on genotypes of the 12-bp indel in intron 1**

In order to evaluate PRNP expression among the genotypes of the 12-bp locus, we selected animals with diplotypes ++/+ (n = 8), ++/+ (n = 8); ++/+ (n = 3); ++/+ (n = 6); ++/+ (n = 5); *significance difference in the PRNP expression (p<0.05).

**PRNP expression with respect to alleles + and − of the 23-bp promoter indel**

Expression of PRNP with respect to alleles + and − of the 23-bp locus were assayed using diplotypes ++/++ and ++/− in the JB breed and diplotypes ++/++ and ++/++ in the JBr breed. Three of the four alleles in the diplotypes selected in the JB breed were the same (−/+ +) so that it was possible to compare the expression of PRNP between two alleles. The JBr breed, except...
for the alleles of interest the other three alleles were similar (+/+) in both diplotypes thus possible to detect the expression of PRNP between alleles + and −. The PRNP levels were 0.0492±0.003 and 0.0099±0.009 for the diplotypes −/+− and −−/+− in the JB breed respectively and 0.1419±0.006 and 0.1787±0.015 respectively for the diplotypes +/+++ and −+/++ in the JBr breed (Table 3). For both breeds, the + allele had a lower expression than those with the − allele (Figure 3). The expression was significant between the two diplotypes in the JB breed (p = 0.0003) as well as in the JBr breed (p = 0.0032).

Discussion

Two PRNP loci have been tentatively shown to influence the cBSE incidence in some cattle populations [6,7,10,14,15–17]. The −− genotype at the 23-bp and the 12-bp loci were reported to be overrepresented in cBSE-affected cattle [6,7,10]. The 23-12-haplotype and a few haplotype tagging single nucleotide polymorphisms (htSNPs) were shown to influence association of the 23-bp polymorphisms (htSNPs) may influence association of the haplotype and a few haplotype tagging single nucleotide polymorphisms [6,7,10] overrepresented in cBSE-affected cattle [6,7,10,14,15–17]. The 23-12-bp haplotype has also been reported to be overrepresented in BSE-affected animals than healthy animals previously [6,7,10] showed a lower PRNP level compared to genotypes ++ and +−. The −− genotype could show high PRNP than the other two genotypes due to a possible over-dominance whereby the − allele might be masked by the + allele [21] or other factors including polar over-dominance as reported in other species [22].

We genotyped the 23-bp and 12-bp indel loci in healthy JB and JBr breeds and analysed the expression of its messenger RNA using ten genotype combinations (five diplotypes in JB and five in JBr). The genotypic and allelic data at these loci supplement the data available in Japan for these and other breeds [11,13,18]. These data were used in the PRNP expression assays and have not been submitted for publication. Genotypic and allelic frequencies data agree with those published in earlier reports. Notably, the allelic frequencies at the 23-bp locus for the JBr breed resembled to those of the United States (US) HF [19] and to those of the healthy Germany cattle [6]. Allelic frequencies at the 12-bp locus in our breeds were in the same range as those reported for US sires [14], US HF [19] and the United Kingdom (UK) HF [7].

We used the diplotypes to study the effect of the genotypes of the 23-bp and 12-bp loci in the PRNP expression. Firstly, we have shown that diplotypes with the −− genotype had higher expression than those with the +− and ++ genotypes at the 23-bp locus in both breeds. Secondly, we showed that diplotypes with the +− genotype had a greater expression than those with the ++ and −− genotypes at the 12-bp locus in both breeds. Considering the genotypes alone, our results suggest the order of PRNP expression as −−/+−/+− at the 23-bp locus and ++/+−/+− at the 12-bp locus in the medulla oblongata of these breeds. Thirdly, we studied the difference of PRNP expression between diplotypes differing in one allele (the + and the −) at the 23-bp locus and showed that diplotypes with the + allele had lower PRNP levels compared to those with the − allele.

In summary, the −− genotype was correlated with an increased PRNP expression compared to ++ and +− genotypes at the 23-bp locus. This genotype was reported to be significantly overrepresented in BSE-affected cattle presumably due to the homozygous deletion of a PRNP transcriptional RP58 binding site [6,7,10,20] at this locus. Surprisingly, the −− genotype at the 12-bp locus which has also been reported to be overrepresented in BSE-affected animals than healthy animals previously [6,7,10] showed a lower PRNP level compared to genotypes +− and ++. The −− genotype could show high PRNP than the other two genotypes due to a possible over-dominance whereby the − allele might be masked by the + allele [21] or other factors including polar over-dominance as reported in other species [22]. There is limited or no information on over-dominance of the + allele at the 12-bp locus in the cattle PRNP but compared to the other two genotypes, the +− genotype has been reported to be high in frequency at the 12-bp locus in many cattle populations [17–19]. Taking the two loci together, our results thus suggest the −− genotype of the 23-bp locus might contribute a greater PRNP level compared to the −− of the 12-bp locus in animals with the −−/−− diplotype as we previously reported [23]. This might be due to the fact that these loci are not entirely independent as they are closely positioned in the region of high LD of the bovine PRNP [15]. Thus if proportional to the expression of PrP, the levels of PRNP may elucidate the levels of PrP in the medulla oblongata of these animals. We further showed that a different between the + and − alleles at the same position in two diplotypes might significantly affect the level of PRNP. Our results showed that an increase of the − allele increased the level of PRNP in one of the two diplotypes examined for expression in alleles. If PRNP expression is an important factor for the PrP expression, animals with genotypes

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**Figure 3. Expression of PRNP** (mean±SE) with respect to alleles + and − of the 23-bp indel in A: JB and B: JBr breeds. +−/+− (n = 20); −−/−− (n = 20); +/+++ (n = 8); +−/++ (n = 7); *significance difference in the PRNP expression (P<0.05). doi:10.1371/journal.pone.0018787.g003
+- and -- at the 23-bp locus may show higher levels of PrP compared to those with the ++ genotype at this locus. It is worth to note that, the +/+ ++ diploype had a low PRNP level compared to the other diploype in the JB breed an indication that the ++ of both loci may show a low PRNP expression. The + variants of either regulatory element have the potential to lower host PrP expression levels [10], thus providing a biological basis for BSE resistance in cattle homozygous for the + [7,24,25]. Our expression results agree with those reported for other tissues in other populations in vivo and also in the in situ [10].

We report here that the 23-bp allele and the -- genotype may contribute to a greater PRNP expression in the 23/12 diploype based assays in the medulla oblongata of JB and JBr breeds. However, the deletion alleles are not entirely independent of one another as there is high linkage disequilibrium (LD) between the two polymorphic sites in Bos taurus cattle populations [15,20]. This suggests that the possible effects of variations in the PRNP on incidence of cBSE may be better understood if PRNP haploype were considered in testing for association with disease incidence. Moreover, PRNP haploypes, containing one or both of the two indel loci, may have a stronger association with either susceptibility or resistance to cBSE than if the indels are considering independently. Also, other functional variants may affect the expression of PRNP and therefore carrying out confirmatory studies in these loci or other polymorphisms using the same tissue or other tissues in other populations would be useful. We performed the assays in individual breeds and did not attempt to compare the breeds due to possible breed effect as we previously reported [23]. We elected to analyze PRNP expression in these breeds because of their economic and socio-cultural importance in Japan. Our sampling places (Kagoshima and Kumamoto prefectures) were preferred because they are the number one producers of JB and JBr breeds respectively. However, there are only a few BSE cases in JB breeds (3 animals in Kumamoto) but the disease has not been diagnosed in JBr breed.

Materials and Methods

Animals, RNA isolation, cDNA synthesis and DNA isolation

We used the medulla oblongata tissue sampled from healthy 120 JB and 98 JBr from public slaughterhouses in Kagoshima and Kumamoto, Japan. Samples were taken randomly, but later coded on the sampling day. Information of these animals was known through a Japanese traceability system at http://www.nibc.go.jp.

Construction of a standard curve and real-time reverse-transcription PCR assay

Real-time PCR standard curves for each of the PRNP and an endogenous control actin beta gene (ACTB) were constructed by PCR products including the target sequence and its flanking sequence as a template. 10-fold dilution series of each standard was used to generate the real-time PCR standard curve. The PCR used to amplify this standard was carried out in the GeneAmp® PCR System 9700 using primer pairs listed in Table 4. Gene expression levels of the bovine PRNP and ACTB were quantitatively measured by the real-time PCR using primer pairs also listed in Table 4. Primer pairs were designed from the Ural sequences GenBank accession numbers JAF98878 (PRNP) and NM_173979 (ACTB) using Primer3 software [26]. The amplified bovine DNAs PRNP 153-bp and ACTB 169-bp were sequenced and confirmed to be identical with those from which primer pairs were designed. The real-time PCR was carried out in a 15-μl reaction volume containing 7.5-μl of SYBR® Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan), 0.03-μM of each primer and 3.0-μl of each cDNA in the 7300 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for one minute. All reactions (standard, unknown samples and non-template control) were performed in duplicates on the same 96-well plate. Results reported here are averages of the duplicates. The PRNP expression level was normalized by dividing it with the ACTB expression level. ACTB was favored against GAPDH because the latter performed poorly in our medulla oblongata samples.

Statistical analyses

Raw data were developed by spreadsheets and later subjected to the GLM procedure of SAS® Proprietary Software, Release 8.2

Concentration and purity of RNA, mRNA and the DNA were calculated by a GeneQuant 100 (GE Healthcare, UK) according to the manufacturer’s instructions.

PRNP genotyping and diplotype analysis

Genotyping with respect to the 23-bp indel within the promoter region and 12-bp indel within the first intron were amplified by polymerase chain reaction (PCR) using primer pairs GTGCG-CAGCGCATGTAAGTG and TGGACAGGGCAATGGG (23-bp indel) also CGCCTTTTACCCCTTCGTG and CACCTT-CACAGATGTGACACCA (12-bp indel). PCR was carried out in a final volume of 20 μl containing 0.5 units of Ex Taq (TAKARA), 40 ng of genomic DNA, 200 mmol/l each of the four deoxynucleoside triphosphates (dNTPs), 10 pmoles of each primer and 2.0 μl of 10× Ex Taq buffer (TAKARA). The mixture was first treated at 94°C for 2 min, followed by 28–35 cycles of denaturation at 94°C for 30 s, annealing temperature 56°C (23-bp); 62°C (12-bp) for 30 s, and extension at 72°C for 30 s and a final elongation step at 72°C for 5 min. All PCRs were carried out in the GeneAmp PCR System 9700 (Applied Biosystems). PCR products were separated on 10% polyacrylamide gels and visualized with ethidium bromide staining under ultra-violet (UV) light. From the genotypes, diplotypes for the two loci were constructed. Animals with the selected diplotypes were used in the gene expression analysis. The diplotypes were selected based on the objectives of our studies while considering the sample sizes. To avoid analysis of animals of the same origin (family relations, sex, age or farms), sampling of the medulla oblongata tissue was restricted to 10–15 samples per a sampling day. Information of these animals was known through a Japanese traceability system at http://www.nibc.go.jp.
Our analyses where composite, with a total of 10,395 records. The data includes bovine prion protein gene (PRNP) expression, sex, age, region/farm and their interactions as shown below. We performed the independent t-tests to support the PRNP expression of the normally distributed data. All decisions were made following the differences in dependent variables but also considered the values of coefficient of determination (R²) and the t grouping. R² values ranging from 0.6725–0.9085 and P-values<0.05 were considered significant in our analyses.

\[ Y_{ijkl} = \mu + D_{i} + S_{j} + A_{k} + L_{l} + D_{x}S_{ij} + D_{x}A_{ik} + D_{x}L_{jl} + \ldots + E_{ijkl} \]

Where as :

- \(Y_{ijkl}\) = Expression
- \(\mu\) = Overall mean
- \(D_{i}\) = Random effect of the \(i^{th}\) diplotype
- \(S_{j}\) = Random effect of the \(j^{th}\) sex

**Table 4.** List of primers used in this study.

| Genes       | sequence                                      | Size | AT °C | Remarks               |
|-------------|-----------------------------------------------|------|-------|-----------------------|
| PRNP        | F 5′-TCCACAGACACAAATCCCA-3′                   | 153  | 60    | External, construction of standard curve |
|             | R 5′-ATCTTCTCCAGGTTTTGTG-3′                   |      |       |                       |
|             | F 5′-TCAACCTGACGTAATCCA-3′                   | 153  | 60    | Internal, real-time PCR|
|             | R 5′-CAGTGTGGTTGCTCGTTCG-3′                  |      |       |                       |
| ACTB        | F 5′-ACCATGGACCCGGCATC-3′                    | 169  | 60    | External, construction of standard curve |
|             | R 5′-TTGCGATCCACATCTGTCG-3′                  |      |       |                       |
|             | F 5′-ATGGAGAACAGATGACGAA-3′                  | 154  | 60    | Internal, real-time PCR|
|             | R 5′-CACATCGTCGATCAATGAAG-3′                 |      |       |                       |

**References**

1. Windl O, Dempster M, Entehej JP, Lathe R, de Silva R, et al. (1996) Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic analysis of predisposing mutations and allelic variation in the PRNP gene. Hum Genet 98: 259–264.
2. Collinge J, Beck J, Campbell T, Entehej K, Will RG (1996) Prion protein gene expression in new variant cases of Creutzfeldt-Jakob disease. Lancet 348: 56.
3. Carlson GA, Goodman PA, Lovett M, Taylor BA, Marshall ST, et al. (1988) Genetic and polymorphism of the mouse prion gene complex: control of scrapie incubation time. Mol Cell Biol 8: 5528–5540.
4. Hunter N, Cairns D, Foster JD, Smith G, Goldmann W, et al. (1997) Is scrapie incubation time a heritable trait? Mamm Genome 8: 553–556.
5. Prusiner S, Hsiao K (1994) Human prion diseases. Ann Neurol 35: 385–395.
6. Prusiner S, Hsiao K (1994) Human prion diseases. Annu Rev 35: 385–395.
7. Brunelle BW, Greenlee JJ, Seabury CM, Keeler JWJ, Smith TPLT, Harzly GP, et al. (2006) Prion gene haplotypes of U.S. cattle sires for potential association with BSE. Mamm Genome 15: 828–833.
8. Murdoch BM, Clawson ML, Yue S, Bass U, McKay S, et al. (2010) PRNP haplotype Associated with Classical BSE Incidence in European Holstein Cattle. PLoS ONE 5: e12786.
9. Seabury CM, Womack JE, Piedrahita J, Derr JN (2004) Comparative PRNP genotyping of U.S. cattle sires for potential association with BSE. Mamm Genome 15: 828–833.
10. Murdoch BM, Womack ML, Yue S, Bass U, McKay S, et al. (2010) PRNP Haplotype Associated with Classical BSE Incidence in European Holstein Cattle. PLoS ONE 5: e12786.
11. Brunelle BW, Keeler Jr. ME, Stabel JR, Moody Sparlock D, Hansen LB, et al. (2008) Allele, Genotype, and Haplotype Data for Bovine Spongiform Encephalopathy-Resistance Polymorphisms from Healthy US Holstein Cattle. PLoS ONE 5(9): e13639.
12. Msalya G (2008) Molecular genetic studies on the prion gene of cattle (susceptibility and/or resistance to BSE). M.Sc. Dissertation, United Graduate School of Agriculture, Kagoshima University, Japan.
13. Shimogiri T, Msalya G, Myint SL, Okamoto S, Kawabe K, et al. (2010) Allele distributions and frequencies of the six prion protein gene (PRNP) polymorphisms in Asian native cattle, Japanese breeds, and mythun (Bos frontalis). Biochim Genet 48: 829–839.
14. Seabury CM, Womack JE, Piedrahita J, Derr JN (2004) Comparative PRNP genotyping of U.S. cattle sires for potential association with BSE. Mamm Genome 15: 828–833.
15. Clawson MLM, Heaton MP, Keeler JWJ, Smith TPLT, Harzly GP, et al. (2006) Prion gene haplotypes of U.S. cattle. BMC Genet 7: 31.
16. Murdoch BM, Clawson ML, Yue S, Bass U, McKay S, et al. (2010) PRNP Haplotype Associated with Classical BSE Incidence in European Holstein Cattle. PLoS ONE 5: e12786.
17. Brunelle BW, Greenlee JJ, Seabury CM, Brown II CE, Nicholson EM (2008) Frequencies of polymorphisms associated with BSE resistance differ significantly between Bos taurus, Bos indicus, and composite cattle. BMC Vet Res 4: 36.
18. Msalya G, Shimogiri T, Okamoto S, Kawabe K, Minesawa M, et al. (2009) Gene and haplotypes polymorphisms of the Prion gene (PRVP) in Japanese Brown, Japanese native and Holstein cattle. Anim Sci J 80: 520–527.
19. Murdoch BM, Womack ML, Yue S, Bass U, McKay S, et al. (2010) PRNP Haplotype Associated with Classical BSE Incidence in European Holstein Cattle. PLoS ONE 5: e12786.
20. Brunelle BW, Keeler Jr. ME, Stabel JR, Moody Sparlock D, Hansen LB, et al. (2008) Allele, Genotype, and Haplotype Data for Bovine Spongiform Encephalopathy-Resistance Polymorphisms from Healthy US Holstein Cattle. J Dairy Sci 91: 333–342.
21. Falconer DS (1961) Introduction to Quantitative Genetics. Oliver and Boyd, Edinburgh and London UK. pp 54–67.
22. Cockett NE, Jackson SP, Shay TL, Farnir F, Berghmans S, et al. (1996) Polar overdominance at the ovine callipyge locus. Science 273: 236–238.

23. Msalya G, Shimogiri T, Nishitani K, Okamoto S, Kawabe K, et al. (2010) Indels within promoter and intron 1 of bovine prion protein gene modulate the gene expression levels in the medulla oblongata of two Japanese cattle breeds. Anim Genet 41: 218–221.

24. Carlson GA, Ebeling C, Yang SL, Telling G, Torchia M, et al. (1994) Prion isolate specified allotypic interactions between the cellular and scrapie prion proteins in congenic and transgenic mice. Proc Natl Acad Sci USA 91: 5690–5694.

25. Manson JC, Clarke AR, McBride PA, McConnell I, Hope J (1994) PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegener 3: 331–340.

26. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, 132, 365–386.