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Short Communication: The double deletion diplotype showed low levels of prion protein at two indel loci of *PRNP* in the medulla oblongata of Japanese Brown cattle

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Msalya, G., Shimogiri, T., Okamoto, S., Kawabe, K. and Maeda, Y. 2012. **Short Communication: The double deletion diplotype showed low levels of prion protein at two indel loci of *PRNP* in the medulla oblongata of Japanese Brown cattle**. Can. J. Anim. Sci. **92**: 153–157. Transmissible spongiform encephalopathies (TSEs) are a class of fatal neurodegenerative diseases caused by abnormally folded prion proteins (PrP). The PrP is necessary for the transmission and propagation of TSE diseases. In this study, PrP was quantified in the medulla oblongata of 39 Japanese Brown (JBr) animals that were genotyped for two indels in the *PRNP* gene – a 23 bp deletion in the promoter region and a 12 bp deletion in the first intron. The mean level of PrP was greater in the ++/++ diplotype than in −−/−− and +−/+− diplotypes, although the differences were not significant. These results suggest that the amount of PrP in the medulla oblongata of animals is related to these indels. However, given that there have been no reported cases of BSE in Japanese Brown animals, the relationship of the indels and PrP levels with the incidence of BSE is unclear.

Key words: Double deletion diplotype, prion protein, indel, medulla oblongata, Japanese Brown cattle

Msalya, G., Shimogiri, T., Okamoto, S., Kawabe, K. et Maeda, Y. 2012. **Communication Brève: Le diplotype à double délétion révèle une faible concentration de prion aux deux locus indel du *PRNP* dans le bulbe rachidien des bovins Bruns japonais**. Can. J. Anim. Sci. **92**: 153–157. Les encéphalopathies spongiformes transmissibles (EST) sont une famille de maladies neurodégénératives mortelles engendrées par des prions, une protéine, anormalement repliés (PrP). Les EST ne peuvent se transmettre ni se propager sans PrP. Dans le cadre de leur étude, les auteurs ont quantifié les PrP dans le bulbe rachidien de 39 bovins Bruns japonais dont le génotype comprenait les locus indel sur le gène *PRNP* – une délétion de 23 paires de bases dans la région promotrice et de 12 paires de bases dans le premier intron. La concentration moyenne de PrP était plus importante dans le diplotype ++/++ que dans les diplotypes −−/−− et +−/+−, bien que l'écart ne soit pas significatif. Ces résultats laissent croire que la quantité de PrP dans le bulbe rachidien des animaux dépend des locus indel. Néanmoins, puisqu'aucun cas d'EST n'a été signalé chez les bovins Bruns japonais, le lien que les locus indel et la concentration de PrP entretiennent avec l'incidence de l'EST n'est pas clair.

Mots clés: Diplotype à double délétion, prion, locus indel, bulbe rachidien, bovins Bruns japonais

The presence of the prion protein (PrP) is necessary for the transmission and propagation of prion diseases, also called transmissible spongiform encephalopathies (TSEs). These fatal neurodegenerative diseases are caused by template refolding of normal cellular host prion proteins (PrP^c) into an abnormal (PrP^{Sc}) infectious form. TSEs can be manifested through acquired, inherited, or sporadic origins (Prusiner 1998). According to Sander et al. (2005), increasing amounts of PrP in the nervous system are associated with a reduction in the incubation period when an animal succumbs to the disease.

Bovine spongiform encephalopathy (BSE) is the TSE of cattle. Classical BSE (cBSE) is the most common type of bovine TSE, with at least 190 500 cases reported in

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Abbreviations: **AU**, absorbance units; **BSE**, bovine spongiform encephalopathy; **BTA**, Bos taurus; **cBSE**, classical BSE; **H-type**, higher molecular mass type BSE; **JB**, Japanese Black cattle; **JBr**, Japanese Brown cattle; **L-type**, lower molecular mass type BSE; **mAU**, milli-absorbance units; ***PRNP***, prion protein gene; **PrP**, prion protein; **PrP^c**, cellular prion protein; **PrP^{Sc}**, Scrapie prion protein; **TSE**, transmissible spongiform encephalopathy; **vCJD**, creutzfeldt-Jakob; −−/−−, double deletion homozygous +−/+−, double heterozygous; ++/++, double insertion homozygous; −−, homozygous deletion; ++, insertion/insertion; +−, insertion/deletion; −−, deletion/deletion

26 countries (http://www.oie.int/eng/info/en_esbmonde.htm). This disease is most likely spread among cattle through the ingestion of BSE-contaminated meat and bone meal. Classical BSE is most likely the cause of the human TSE known as variant Creutzfeldt-Jakob disease (vCJD) (Collinge et al. 1996). Two additional isoforms of BSE have been described in the literature: (1) a type with a lower molecular mass of the unglycosylated isoform (L-type) and (2) a type with higher molecular mass of the unglycosylated isoform (H-type). The H and L types of BSE are rare and are collectively referred to as atypical BSE, with reports in several countries (Brown et al. 2006).

The prion protein gene (*PRNP*) instructs the formation of PrP. It is expressed in the brain and other tissues and encodes a protein of about 250 amino acids (Stahl et al. 1993). In cattle, *PRNP* is located on BTA13q17 and is made up of three exons (Fig. 1). Other loci may have a considerable effect on the susceptibility of cattle to BSE (Zhang et al. 2004; Juling et al. 2008).

Polymorphisms in *PRNP* have been shown to influence the susceptibility of TSEs and their incubation periods in humans (Shibuya et al. 1998), sheep (Westaway et al. 1994), goats (Goldmann et al. 1996) and mice (Moore et al. 1998). Polymorphisms in the 5′ region of the bovine *PRNP* have been associated with either resistance or susceptibility of cattle to BSE (Sander et al. 2004; Juling et al. 2006). To date, there are many reports of polymorphisms in the bovine *PRNP* (Hills et al. 2001; Brunelle et al. 2008; Msalya et al. 2009) and their relationship with BSE (Sander et al. 2004; Juling et al. 2006). Expression of the bovine *PRNP* has also been characterized (Sander et al. 2005; Xue et al. 2008). We (Msalya et al. 2010, 2011) previously reported that bovine *PRNP* is highly expressed in animals that are double deletion homozygotes (−−/−−) for 23-bp and 12-bp insertion/deletion (indel) in the promoter and first intron, respectively, of the *PRNP* gene. The present study was designed to evaluate the levels of PrP in the medulla oblongata of Japanese Brown (JBr) cattle with different diplotypes for the 23-bp and 12-bp indels. Although there is no reported BSE in the JBr breed (http://www-bm.mhlw.go.jp/english/topics/foodsafety/bse), the frequencies of these mutations are generally higher in JBr [23-bp- =0.71; 12-bp- =0.67 (Msalya et al. 2009, 2010)] than in Japanese Black (JB) cattle [23-bp- =0.59; 12-bp- =0.57 (Nakamitsu et al. 2006)]. We therefore elected to use JBr, the second largest beef cattle breed in Japan. JBr cattle were introduced into Japan from Korea in the 1800s and approved as a breed after extensive crossbreeding with Simmental and Devon animals (Nishida 1973). However, JBr is facing a reduction in numbers due to competition from profitable breeds like the JB breed (Honda et al. 2006).

PrP was analyzed in 1-mm pieces of medulla oblongata collected from 39 slaughtered JBr animals (Table 1) that were randomly selected from a public slaughter house in the Kumamoto region. The animals had been raised by private farmers and tested as BSE negative at the slaughter house using ELISA assay (“Platelia” ELISA-kit; Bio-Rad Laboratories; http://www.mofa.go.jp/region/n-america/us/bse0407.pdf). In addition to PrP protein levels, the samples were also genotyped for the *PRPN* 23-bp and 12-bp indels (Fig. 1). Although these samples were not included in our previous studies, the DNA genotyping, diplotype analysis, sampling procedure and processing were as previously described by Msalya et al. (2010, 2011). The experiments in this study comply with the current animal welfare laws of Japan and neither humans nor animals were harmed during the course of study and experiments.

Levels of PrP were determined in crude protein extracts from the medulla oblongata of the 39 animals using a double-antibody sandwich technique from an enzyme immunoassay kit (SPI-Bio, France) according to the manufacturer’s instructions. PrP was detected as the amount of light absorbed by the sample (absorbance units) at 405 nm wavelength in a Mithrus LB 940 microplate reader (Bethrod technologies GmbH, Germany). Two AU readings were recorded for each sample. Availability and concentration of PrP were determined by a GeneQuant 100 (GE Healthcare, UK). Raw data were developed by using microwin 2000 software (Mikrotek Laborsysteme GmbH) before further analyses. The reading of a blank well was subtracted from readings of the target samples before data analysis. The three diplotypes used in this study [double insertion homozygous (++/++), double heterozygous indel (+−/+−) and double deletion homozygous (−−/−−)] coded by age and sex were identified as described by Msalya et al. 2010 (Fig. 2). Statistical analysis was performed with the GLM procedure of SAS PROPRIETARY Software, Release 8.2 (SAS Institute, Inc.) using a linear model that included diplotype, sex, age and their interactions.

A total of 39 JBr animals, equally divided between the three diplotypes (++/++, +−/+−, and −−/−−), were included in the analysis. Levels of PrP are

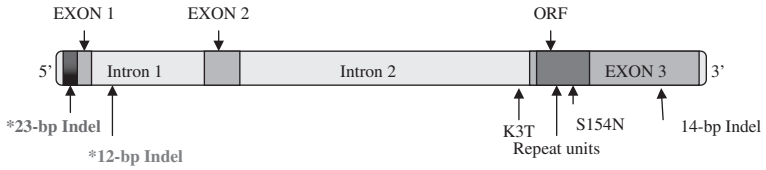

Fig. 1. Organization and polymorphisms of the bovine *PRNP* (*Loci analyzed in this study).

Table 1. JBr animals used in the analysis of PrP

Diplotype	++/++	+−/+−	−−/−−
Sex		Age (months)	
Female	27	26	27
	25	26	27
	25	25	27
	25	25	24
	24	24	24
	24	24	24
		24	
Male	27	25	26
	27	25	25
	26	25	25
	25	24	25
	25	24	24
	24	24	24
	24		24

Table 2. Expression of PrP (mAU[z] ± SE) at three diplotypes in selected JBr animals

Diplotype	*n*	Mean PrP	SD[z]	SE[z]
++/++	13	15.66	3.52	0.98
+−/+−	13	13.93	4.01	1.1
−−/−−	13	11.93	4.11	1.1

[z]mAU, mean milli-absorbance unit; SD, standard deviation; SE, standard error.

presented in Table 2 for the three diplotypes as mean (±SE) of the milli-absorbance units (mAU). The mean PrP was 15.66±0.98 in the ++/++ diplotype, compared to 13.93±1.11 in the +−/+− diplotype and 11.93±1.12 in the −−/−− diplotype. However, there was no significance difference in the absorbance of PrP among diplotypes, age, and sex of the animals at the 5% level (Table 3).

It has previously been shown that the −−/−− diplotype is significantly over-represented in BSE-affected animals (Juling et al. 2006), as well as the 23-bp −/− genotype and the 23-/12- haplotype (Sander et al. 2004; Juling et al. 2006). Also, animals with the −−/−− diplotype have been reported to show a greater expression of *PRNP* (Sander et al. 2005; Msalya et al. 2010). Gene expression was higher in −/− genotype than +/+ and +/− at the 23-bp indel, whereas compared with the two homozygous genotypes, the expression was higher in +/− at the 12-bp indel locus (Msalya et al. 2011). We also showed that the ++/++ diplotype had lower *PRNP* expression compared with other diplotypes in Japanese cattle breeds and that the +/+ genotypes of both the 23-bp and 12-bp loci may have a lower *PRNP* expression than the other genotypes. According to Sander et al. (2005), the + variants of both loci have the potential to lower host PrP protein levels, and therefore, may provide a biological basis for BSE resistance in cattle homozygous for the + form (Carlson et al. 1994; Juling et al. 2006). In addition, high amounts of PrP in an animal will most likely reduce the incubation period when the animal

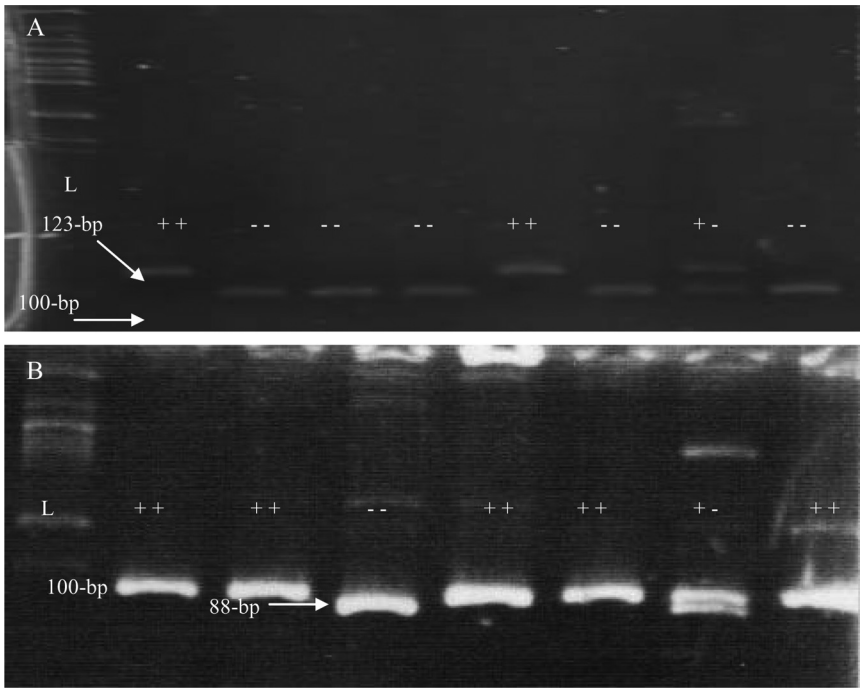

Fig. 2. Appearance of the two loci of the bovine *PRNP* by electrophoresis. A: 23-bp indel B: 12-bp indel. ++: Insertion/insertion; +−: Insertion/deletion; −−: Deletion/deletion (Diplotypes were obtained from combination of these genotypes).

Table 3. ANOVA for the expression of PrP in JBr breed[z]

SoV	DF	SS	MS	F	Pr > F
D	2	90.63	45.31	2.42	0.11
S	1	9.85	9.85	0.53	0.48
Ag	3	67.71	22.57	1.20	0.33
D × S	2	5.72	2.86	0.15	0.86
D × Ag	5	16.90	3.38	0.18	0.97
S × Ag	2	12.91	6.46	0.34	0.71

[z]D, diplotype; S, sex; Ag, age; D × S, diplotype × sex; D × Ag, diplotype × age; S × Ag; sex × age; SoV, source of variation; df, degrees of freedom; SS, sum of squares; MS, mean squares; F, *F*-value; Pr, probability.

succumbs to the prion disease. Moreover, the +/− genotype at the 12-bp locus has been reported to be high in frequency in many cattle populations (Brunelle et al. 2007, 2008; Msalya et al. 2009). Therefore, we elected to determine the levels of PrP in animals with the three diplotypes. Based on previous results for *PRNP* expression, we predicted that the level of PrP would be highest in the −−/−− diplotype or in the 23-bp −/− genotype.

Contrary to the results for *PRNP* expression where *PRNP* was lowest in ++/++ animals, the amount and concentration of PrP protein was numerically higher in the ++/++ diplotype compared with the other two diplotypes (Table 2). However, it should be noted that these differences were not significant. Several possibilities can explain the present findings including: (1) the sample size ($n = 13$) used for each diplotype was small and therefore, not representative, (2) no BSE has been reported in the JBr breed (http://www-bm.mhlw.go.jp/english/topics/foodsafety/bse), and (3) translation may be incomplete in the −−/−− diplotype, resulting in a reduced level of intact PrP in these animals, and therefore the level of *PRNP* expression is not predictive of the level of PrP in that particular diplotype. To investigate these results further, a larger sample size and/or different breeds should be used to determine the expression of PrP. It is also necessary to measure the absorbance in different tissue of animals with different diplotypes. If confirmed, low PrP levels in the JBr animals possessing the −−/−− diplotype may suggest a unique process of *PRNP* transcription within this breed.

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