

The effect of insertion/deletion polymorphisms within the promoter and intron 1 sequences of the PRNP gene on the breeding value of Holstein-Friesian bulls*

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(Accepted January 20, 2015)

The aim of this study was to verify the hypothesis that insertion/deletion (*indel*) polymorphisms within the promoter and intron 1 sequences of the prion protein (PRNP) gene can affect the breeding value of Holstein-Friesian bulls. The experimental material included 261 Holstein-Friesian bulls born between 1997 – 2002. It was shown that the polymorphism in the intron 1 (12 bp *indel*) sequence had a more significant effect on the analyzed traits than a polymorphism in the promoter (23 bp *indel*) sequence. A deletion allele within intron 1 (12del) significantly increased the bulls' breeding value for milk yield ($p=0.001$), protein yield ($p=0.042$), type and conformation ($p=0.018$), udder width ($p=0.003$), dairy character ($p=0.004$), and decreased days open ($p=0.022$). A deletion allele (23del) at the polymorphic locus of the promoter significantly decreased the bulls' breeding value for milk yield ($p=0.001$), udder width ($p=0.029$) and dairy character ($p=0.033$). Analysis of both alleles showed that 23del-12del haplotype increased the bulls' breeding value for udder traits: udder ($p=0.008$), fore udder ($p=0.043$) and udder depth ($p=0.041$), and decreased for fat content ($p=0.047$) and conformation traits - body depth ($p<0.001$), chest width ($p=0.029$) and rear leg set - side view ($p=0.029$). Also positive effect of 23del-12ins haplotype for days open ($p=0.003$) and days between calving and first insemination ($p=0.045$) was observed.

*This work was financially supported by the University of Warmia and Mazury (research project No. 0105-0804).

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KEYWORDS: association / breeding value / Holstein bulls / polymorphism / PRNP gene

Candidate genes associated with production and functional traits are thought to be located on bovine chromosome 13 (BTA13) [Ashwell *et al.* 2004, Cole *et al.* 2011, Mosig *et al.* 2001, Olsen *et al.* 2002, Plante *et al.* 2001, Rupp and Boichard 2003, Schwerin *et al.* 2004]. However, candidate gene markers useful in breeding practice have not been fully identified. The PRNP gene, is considered as a potential candidate gene for Bovine Spongiform Encephalopathy (BSE) susceptibility/resistance [Juling *et al.* 2006], as well as milk production traits [Strychalski *et al.* 2011]. The PRNP gene has been localized on bovine chromosome 13 (BTA13) band q17 (BTA13q17), between microsatellite loci BM1580 (position 49.9 cM) and UWCA25 (50.6 to 52.8 cM) [Schläpfer *et al.* 2000].

Numerous mutations have been identified within the PRNP gene [Clawson *et al.* 2006], but it is believed that *indel* polymorphisms within the 23 bp (23*indel*) promoter sequence and the 12 bp (12*indel*) intron 1 sequence of the PRNP gene might influence BSE incubation time and/or BSE susceptibility in Holstein-Friesian cattle [Sander *et al.* 2004; Juling *et al.* 2006]. It has been demonstrated that the 23 bp *indel* polymorphism within the promoter sequence contains a binding site for the RP58 protein - a strong transcription repressor, while the 12 bp *indel* polymorphism contains a potential binding site for the SP1 transcription factor. A model has been proposed, in which the 23del-12del haplotype affects higher promoter activity and the 23ins-12ins haplotype determines lower gene expression [Sander *et al.* 2005, Msalya *et al.* 2009]. The 23del-12del haplotype is associated with an increased risk of BSE. This haplotype is more frequent in Holstein-Friesian cattle [Brunelle *et al.* 2008, Haase *et al.* 2007, Juling *et al.* 2006, Czarnik *et al.* 2011, Gurgul *et al.* 2012], whereas the 23ins-12ins haplotype is more frequent in endemic and dual-purpose breeds [Haase *et al.* 2007, Jeong *et al.* 2006, Juling *et al.* 2006, Czarnik *et al.* 2009].

Further studies are needed to investigate inter-breed differences in genotype frequencies in particular *indels* within a sequence, and to test the hypothesis regarding functional differences between haplotypes. Research conducted to date suggests that *indel* polymorphisms could be potential markers of traits responsible for breeding value in cattle. Microsatellite markers, genes and regions on chromosome BTA 13 are associated with milk, fat and protein yields [Ashwell *et al.* 2004, Cole *et al.* 2011, Mosig *et al.* 2001, Olsen *et al.* 2002, Plante *et al.* 2001, Rupp and Boichard 2003]. Previous association studies have revealed that *indel* polymorphisms affect the milk performance traits of dairy cows [Czarnik *et al.* 2007, Strychalski *et al.* 2011, Rzewucka-Wójcik *et al.* 2013], blood lymphocyte profile and immune system's efficiency [Kaczmarczyk *et al.* 2010].

The objective of this study was to verify the hypothesis that *indel* polymorphisms within the promoter (23*indel*) and intron 1 (12*indel*) sequences of the PRNP gene can affect the breeding value of Holstein-Friesian bulls.

Material and methods

The experimental material comprised of 261 randomly selected Holstein-Friesian bulls, the offspring of 68 sires, born in 1997 – 2002. The breeding value of bulls was estimated on basis of 30 traits (at least 90 daughters) included in a freely accessible database of the National Research Institute of Animal Production in Cracow (<http://www.izoo.krakow.pl>). The sire evaluation was performed in December 2013.

Genomic DNA was isolated from peripheral blood leukocytes using the Master Pure™ Purification Kit (EPICENTRE Biotechnologies, Madison, WI, USA). *Indel* polymorphisms at the PRNP gene were determined by PCR. The amplified sequences included two fragments of the PRNP gene (GenBank AJ298878), located within its promoter (position 47784 to 47883 bp) and intron 1 (position 49686 to 49777 bp). PCR primers were used for amplification, as described by Sander et al. (2004). PCR products were electrophoresed in 1.5% AmpliSize agarose gel (Bio-Rad Laboratories, CA, USA). Polymorphisms were visualized by Fluor S™ Multimager (Bio-Rad Laboratories, CA, USA).

The effect of PRNP alleles on the breeding value of bulls was estimated using the following linear mixed model equation (SAS/STAT Version 9.1, SAS Institute Inc., Cary, NC, USA):

$$y = X_1\mu + X_2\beta + Z\alpha + e$$

where:

- y – denotes vector of the deregressed estimated bull's breeding value for one of the 30 traits routinely evaluated in Polish Holstein Bulls (milk performance, conformation, fertility traits, indexes, SCC and longevity) at the National Research Institute of Animal Production, Balice (evaluation 08/2012);
- μ – the overall mean;
- $\alpha \sim N(0, A\sigma_a^2)$ – the vector of random additive polygenic effects of bulls with A being an additive polygenic relationship matrix and σ_a^2 being an additive variance;
- β – the vector of fixed additive effects of *indel* PRNP polymorphisms;
- $e \sim N(0, I\sigma_e^2)$ – the vector of residual effects with I being a diagonal matrix with elements on diagonal representing the reciprocal of the accuracy of the estimated deregressed breeding value for each bull;
- σ_e^2 – residual variance;
- X_1 , X_2 and Z – the design matrices for μ , β and α , respectively.

The parameters of this model were estimated by solving Mixed Model Equations (as implemented in the SAS 9.1 proc mixed) with variances assumed as known and amounting to $\sigma_a^2 = 0.3\sigma_y^2$ and $\sigma_e^2 = 0.7\sigma_y^2$ (where σ_y^2 is a phenotypic variance).

To evaluate significance of differences between groups, test t was used.

Haplotype frequencies and haplotype-trait associations were calculated using

EM algorithm and GLM method included in „hapassoc” package [Burkett *et al.* 2006] written to R software [R Development Core Team 2011].

Results and discussion

Frequency of genotypes, alleles and haplotypes determined by the *indel* polymorphism at the PRNP gene in the analyzed bulls is presented in Table 1. The heterozygous genotype (ins/del) was most frequent at both examined polymorphic *loci* of the PRNP gene (23ins/del – 0.525, 12ins/del – 0.529), intermediate values were recorded for del/del homozygotes (23ins/del – 0.352, 12ins/del – 0.291), while ins/ins homozygotes were the least frequent (23ins/del – 0.123, 12ins/del – 0.180). An insertion allele (23ins – 0.385, 12ins – 0.445) was less frequent at both polymorphic *loci*. Four haplotypes were found. The highest frequency was observed for 23del-12del (0.549) and 23ins-12ins (0.377) haplotypes, whereas 23del-12ins (0.066) and 23ins-12del (0.008) frequencies were low.

Table 2 shows the effect of *indel* polymorphisms within the PRNP gene on the breeding value of 261 Holstein-Friesian bulls routinely estimated for milk production traits, body conformation traits and fertility traits. A polymorphism in the intron 1 (12*indel*) sequence had a more significant effect on the analyzed traits than a polymorphism then the promoter (23*indel*) sequence. A deletion allele within intron 1 (12del) significantly increased the breeding value of bulls with respect to two production traits: milk yield (by 187.27±56.74 kg; p=0.001) and protein yield (by 4.50±2.20 kg; p=0.042), and three body conformation traits: type and conformation (by 2.28±0.95 points; p=0.018), udder width (by 3.12±1.04 points; p=0.003) and dairy character (by 2.77±0.95 points; p=0.004), whereas a statistical significant tendency (p<0.1) was noted for udder and overall conformation. In the group of fertility traits, a significant effect of reducing the value was observed for days open (-4.65±2.02 points; p=0.022), and a statistical significant tendency (p<0.1) – for days between calving and first insemination. A deletion allele (23del) at the polymorphic *locus* of the promoter significantly decreased the bulls’ breeding value for one production trait: milk yield (by -191.74±59.35; p=0.001), and two body conformation traits: udder

Table 1: Genotypic, allelic and haplotypes frequencies of 23 bp and 12 bp *indels* of PRNP gene in investigated Holstein-Friesian bulls

Region of PRNP gene	N	Genotype frequency		Allele frequency		Haplotype frequency			
		ins/ins	ins/del	del/del	ins	del	23del-12del	23ins-12ins	23ins-12del
Promoter	261	0.123	0.525	0.352	0.385	0.615			
23 bp <i>indel</i>	261	0.180	0.529	0.291	0.445	0.555	0.549	0.377	0.066
Intron 1									
12 bp <i>indel</i>									0.008

PRNP gene polymorphisms and breeding value of bulls

Table 2. Estimated additive effects of 23del and 12del alleles at the PRNP gene locus with regards to deregressed breeding value of Holstein-Friesian bulls

Trait	Polymorphic site	Allele	Estimated effect	SE	p value
Milk yield (kg)	23indel	Del	-191.74	59.35	0.001
	12indel	Del	187.27	56.74	0.001
Fat yield (kg)	23indel	Del	1.64	2.58	0.527
	12indel	Del	-2.66	2.47	0.282
Fat content (%)	23indel	Del	-0.01	0.13	0.941
	12indel	Del	-0.13	0.13	0.315
Protein yield (kg)	23indel	Del	-4.00	2.30	0.083
	12indel	Del	4.50	2.20	0.042
Protein content (%)	23indel	Del	-1.66	1.29	0.201
	12indel	Del	1.94	1.24	0.117
Size (pts)	23indel	Del	-1.36	1.06	0.201
	12indel	Del	1.36	1.01	0.180
Type and conformation	23indel	Del	-1.81	0.10	0.070
	12indel	Del	2.28	0.96	0.018
Feet and legs	23indel	Del	-0.27	1.30	0.833
	12indel	Del	0.87	1.24	0.482
Udder	23indel	Del	-0.33	1.00	0.745
	12indel	Del	1.77	0.96	0.066
Overall conformation (pts)	23indel	Del	-0.78	0.95	0.413
	12indel	Del	1.69	0.90	0.062
Stature	23indel	Del	-1.56	1.17	0.184
	12indel	Del	1.63	1.11	0.146
Body depth	23indel	Del	-3.16	1.65	0.057
	12indel	Del	-0.12	1.58	0.940
Chest width	23indel	Del	-1.34	1.83	0.466
	12indel	Del	-1.06	1.75	0.546
Rump angle	23indel	Del	-1.03	1.77	0.562
	12indel	Del	1.30	1.70	0.445
Rump width	23indel	Del	-1.12	1.89	0.553
	12indel	Del	1.39	1.80	0.443
Rear leg set - side view	23indel	Del	-0.28	1.78	0.874
	12indel	Del	-1.72	1.71	0.315
Foot angle	23indel	Del	-0.74	1.64	0.653
	12indel	Del	-0.36	1.57	0.817
Rear leg set - rear view	23indel	Del	-1.17	1.86	0.528
	12indel	Del	0.75	1.77	0.672
Fore udder	23indel	Del	0.68	1.61	0.674
	12indel	Del	0.91	1.54	0.555
Rear udder height	23indel	Del	0.96	9.69	0.921
	12indel	Del	6.86	9.26	0.460
Udder support (central ligament)	23indel	Del	-0.76	1.37	0.581
	12indel	Del	1.85	1.31	0.160
Udder depth	23indel	Del	1.66	1.56	0.288
	12indel	Del	0.91	1.49	0.544
Udder width (pts)	23indel	Del	-2.38	1.08	0.029
	12indel	Del	3.12	1.04	0.003
Front teat placement	23indel	Del	0.18	1.45	0.900
	12indel	Del	0.81	1.39	0.557
Dairy character	23indel	Del	-2.15	1.00	0.033
	12indel	Del	2.77	0.96	0.004
Non-return rate at 56 days at first insemination, heifer	23indel	Del	-1.24	1.72	0.473
	12indel	Del	0.43	1.64	0.794
Non-return rate at 56 days at first insemination, cow	23indel	Del	-1.50	1.86	0.420
	12indel	Del	1.82	1.77	0.306
Days between calving and first insemination	23indel	Del	2.51	2.04	0.219
	12indel	Del	-3.61	1.95	0.065
Days open	23indel	Del	4.07	2.11	0.055
	12indel	Del	-4.65	2.02	0.022
Somatic cell count	23indel	Del	0.90	1.76	0.610
	12indel	Del	-0.17	1.68	0.919

Significant effects are marked as bold letters.

Table 3. Estimated effects of 23del-12del and 23del-12ins haplotypes at the PRNP gene locus with regards to deregressed breeding value of Holstein-Friesian bulls

Trait	Haplotype	Estimated effects*	SE	p value
Milk yield (kg)	23del-12del	1.48	33.08	0.964
	23del-12ins	-114.93	6.65	0.086
Fat yield (kg)	23del-12del	-0.91	1.44	0.527
	23del-12ins	2.90	2.47	0.443
Fat content (%)	23del-12del	-0.17	0.07	0.047
	23del-12ins	-0.07	0.15	0.647
Protein yield (kg)	23del-12del	0.96	1.23	0.435
	23del-12ins	1.54	2.44	0.528
Protein content (%)	23del-12del	0.01	0.01	0.335
	23del-12ins	0.01	0.02	0.619
Size (pts)	23del-12del	0.09	0.59	0.879
	23del-12ins	-0.24	1.19	0.838
Type and conformation	23del-12del	0.53	0.56	0.348
	23del-12ins	-1.04	1.12	0.357
Feet and legs	23del-12del	0.65	0.73	0.373
	23del-12ins	0.38	1.46	0.797
Udder	23del-12del	1.49	0.56	0.008
	23del-12ins	0.26	1.13	0.815
Overall conformation (pts)	23del-12del	0.98	0.53	0.065
	23del-12ins	0.02	1.07	0.987
Stature	23del-12del	0.18	0.65	0.777
	23del-12ins	-0.12	1.31	0.925
Body depth	23del-12del	-3.18	0.93	<0.001
	23del-12ins	-1.95	1.86	0.295
Chest width	23del-12del	-2.24	1.02	0.029
	23del-12ins	0.65	2.06	0.752
Rump angle	23del-12del	0.46	0.99	0.642
	23del-12ins	1.41	1.99	0.478
Rump width	23del-12del	0.27	1.06	0.797
	23del-12ins	-1.01	2.14	0.635
Rear leg set - side view	23del-12del	-2.17	0.99	0.029
	23del-12ins	-2.50	2.00	0.210
Foot angle	23del-12del	-1.06	0.92	0.250
	23del-12ins	-0.25	1.86	0.892
Rear leg set - rear view	23del-12del	-0.34	1.04	0.747
	23del-12ins	-0.10	2.10	0.962
Fore udder	23del-12del	1.67	0.90	0.043
	23del-12ins	1.82	1.81	0.313
Rear udder height	23del-12del	7.74	5.46	0.156
	23del-12ins	-0.00	10.92	1.000
Udder support (central ligament)	23del-12del	1.07	0.77	0.164
	23del-12ins	-0.96	1.55	0.536
Udder depth	23del-12del	1.80	0.88	0.041
	23del-12ins	3.14	1.77	0.077
Udder width (pts)	23del-12del	0.76	0.61	0.221
	23del-12ins	-2.06	1.23	0.092
Front teat placement	23del-12del	1.00	0.82	0.220
	23del-12ins	0.26	1.64	0.872
Dairy character	23del-12del	0.67	0.56	0.236
	23del-12ins	-1.63	1.13	0.148
Non-return rate at 56 days at first insemination, heifer	23del-12del	-0.81	0.97	0.401
	23del-12ins	-1.33	1.95	0.493
Non-return rate at 56 days at first insemination, cow	23del-12del	0.34	1.05	0.746
	23del-12ins	-1.28	2.10	0.544
Days between calving and first insemination	23del-12del	-0.94	1.14	0.409
	23del-12ins	4.59	2.29	0.045
Days open	23del-12del	-0.36	1.17	0.757
	23del-12ins	6.92	2.35	0.003
Somatic cell count	23del-12del	0.81	0.99	0.412
	23del-12ins	1.99	1.99	0.318

*Calculated using 23ins-12ins haplotype as a reference category. Significant effects are marked as bold letters.

width (by -2.38 ± 1.08 points; $p=0.029$) and dairy character (by -2.15 ± 0.00 points; $p=0.033$). A statistical significant tendency ($p < 0.1$) towards lower breeding value was noted for protein yield, type and conformation and body depth, while towards higher value for days open.

Table 3 shows the estimated effects of linkage complexes (haplotypes) determined by the mutations in both polymorphic *loci* of PRNP gene. Haplotype 23ins-12del was ignored due to its low frequency in examined group (0.008). Results show that 23del-12del haplotype significantly increased the breeding value for three udder traits: udder (by 1.49 ± 0.56 points; $p=0.008$), fore udder (by 1.87 ± 0.90 points; $p=0.043$) and udder depth (by 1.80 ± 0.88 points; $p=0.041$), and significantly decreased one production trait – fat content (by -0.17 ± 0.07 points; $p=0.047$), three conformation traits – body depth (by -3.18 ± 0.93 points; $p < 0.001$), chest width (by -2.14 ± 1.02 points; $p=0.029$) and rear leg set – side view (by -2.17 ± 0.99 points; $p=0.029$). Haplotype 23del-12ins showed statistically significant effect on fertility traits – days open (by 6.92 ± 2.35 points; $p=0.003$) and days between calving and first insemination (by 6.92 ± 2.29 points; $p=0.003$).

The identification of direct and indirect effects of *indel* polymorphisms at the PRNP gene *locus* is an important biological and breeding consideration. The mechanisms responsible for increased frequency of the del/del genotype in the promoter and intron 1 sequences of the PRNP gene in Holstein-Friesian cattle [Sander *et al.* 2004, Czarnik *et al.* 2011], including BSE-affected animals [Sander *et al.* 2004, Juling *et al.* 2006, Gurgul *et al.* 2012] and animals intensively selected for milk production traits [Seabury *et al.* 2004, Czarnik *et al.* 2011], remain unknown. The PrP is essential for the formation of the RNA particle, in particular domain of PrP contained residues 30 to 49. Recent studies have demonstrated that cytoplasmic PrP expression can induce the formation of large ribonucleoprotein particles and thereby also affect post-transcriptional regulation of overall protein expression. This is evidence for relation between PrP and RNA in cells and confirmed, that cytosolic PrP assembling a large RNA processing centre which have termed PrP-RNP for PrP-induced ribonucleoprotein particle [Beaudoin *et al.* 2009]. It suggests that the PrP affects the expression of numerous genes, including genes whose protein products modify milk performance traits and functional traits in cattle.

Several regions carrying potential QTLs for milk production traits have been identified on bovine chromosome BTA13 near the PRNP locus. The results of genome-wide association studies (GWAS) on single nucleotide polymorphisms (SNP) and an analysis of 31 production, health, reproduction and body conformation traits in contemporary U.S. Holstein cows revealed that the 58 Mb region of BTA13 with four genes near the GNAS gene (guanine nucleotide binding protein, alpha stimulating; position 69.27 cM) was highly significant for milk yield, protein yield and fat yield. The most significant BTA13 SNP effect for strength was ranked seventh for the trait and was in the pitrilysin metalloproteinase 1 gene (PITRM1; position from 47.02 to 47.02 cM), which also was linked to two of the top 20 effects for stature and three of the top 20 effects for body depth [Cole *et al.* 2011].

Few attempts have been made to verify the role of *indel* polymorphisms within the PRNP gene as potential markers of milk production traits in cattle. It was found that the 23 bp *indel* polymorphism within the promoter sequence differentiated the yield and content of protein in the milk of Polish Holstein-Friesian cows, which were highest in 23ins/del heterozygotes and lowest in 23del/del homozygotes [Strychalski *et al.* 2011]. Czarnik *et al.* [2007] noted a correlation between the 23 bp *indel* polymorphism in the promoter region and the concentrations of protein and solids non-fat in the milk of cows naturally infected with the bovine leukemia virus (BLV) during the first three months of lactation. The effect of *indel* polymorphisms at the PRNP gene on milk production traits has also been investigated in Jersey cattle. The 12 bp *indel* polymorphism in the intron 1 region was found to affect milk yield and fat content during the second lactation [Rzewucka-Wójcik *et al.* 2013].

In this work, for the first time, the associations between insertion/deletion (*indel*) polymorphisms of the PRNP gene and bulls' breeding value were reported. Obtained results show the opposing effect of analysed deletion (23del and 12del) alleles. The investigated 23del allele decreases, while 12del allele increases the breeding value of analyzed traits. Simultaneous analysis of both alleles (haplotypes) showed that the effects observed for single deletion alleles were mutually neutralized/equalized. Additionally, we showed that presence of both deletion alleles significantly increases the breeding values for udder traits, which weren't recorded during analysis of single mutations.

Obtained results might suggest that in Holstein-Friesian cattle frequent occurrence of the deletion allele in the polymorphic locus in intron 1 PRNP gene in comparison with endemic and dual-purpose breeds might be the induced pre-selection effect that was aimed to improve some udder traits in cows.

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