Original Article

Correlation between the Insertion/Deletion Mutations of Prion Protein Gene and BSE Susceptibility and Milk Performance in Dairy Cows

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Objective To investigate the 23 bp and 12 bp insertion/deletion (indel) mutations within the bovine *prion* protein (PRNP) gene in Chinese dairy cows, and to detect the associations of two indel mutations with BSE susceptibility and milk performance.

Methods Based on bovine PRNP gene sequence, two pairs of primers for testing the 23 bp and 12 bp indel mutations were designed. The PCR amplification and agarose electrophoresis were carried out to distinguish the different genotypes within the mutations. Moreover, based on previous data from other cattle breeds and present genotypic and allelic frequencies of two indels mutations in this study, the correlations between the two indel mutations and BSE susceptibility were tested, as well as the relationships between the mutations and milk performance traits were analyzed in this study based on the statistical analyses.

Results In the analyzed Chinese Holstein population, the frequencies of two “del” alleles in 23 bp and 12 bp indel mutations were more frequent. The frequency of haplotype of 23del-12del was higher than those of 23del-12ins and 23ins-12del. From the estimated r^2 and D' values, two indel polymorphisms were linked strongly in the Holstein population ($D' = 57.5\%$, $r^2 = 0.257$). Compared with the BSE-affected cattle populations from the reported data, the significant differences of genotypic and allelic frequencies were found among present Holstein and some BSE-affected populations ($P < 0.05$ or $P < 0.01$). Similarly, there were significant frequency distribution differences of genotypes and alleles among Chinese Holstein and several previous reported healthy dairy cattle ($P < 0.05$ or $P < 0.01$). Moreover, association of genotype and combined genotypes of two indel polymorphisms with milk performance and resistant mastitis traits were analyzed in Holstein population, but no significant differences were found ($P > 0.05$).

Conclusions These observations revealed that the influence of two indel mutations within the bovine PRNP gene on BSE depended on the breed and they did not affect the milk production traits, which laid the foundation for future selection of resistant animals, and for improving health conditions for dairy breeding against BSE in China.

Key words: Dairy cows; *Prion* protein (PRNP) gene; Bovine spongiform encephalopathy (BSE); Insertion/deletion (indel) mutation; Association; Milk performance

Transmissible spongiform encephalopathy (TSE) is a fatal, infectious, neurodegenerative disease, affecting many species including humans with Creutzfeldt Jakob disease (CJD), variant Creutzfeldt Jakob disease (vCJD), kuru, bovine spongiform encephalopathy (BSE) in cattle, chronic

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wasting disease (CWD) in deer and elk, transmissible mink encephalopathy (TME) in mink, and scrapie in sheep and goats.^{1,2} Moreover, TSE is characterized by accumulation of an abnormal isoform (PrP^{Sc}) in the central nervous system (CNS) of the host-encoded cellular prion protein (PrP^C). The normal cellular prion protein (PrP^C) and PrP^{Sc} are encoded by the same *prion* protein (PRNP) gene, with exactly the same amino acid sequence, but they differ only in their tertiary structure.¹ Mammal PRNP is encoded by a single copy PRNP gene and the entire open reading frame (ORF)

is found within a single exon.^{2,3} Previous studies have reported that the coding variations of the mammalian PRNP gene are associated with TSE susceptibility, but the bovine PRNP coding sequences is excluded.²

In humans, an M129V polymorphism of the PRNP gene coding sequence is strongly correlated with susceptibility to variant CJD.⁴⁻⁷ In sheep, the polymorphisms, such as V136A, R154H, Q171R/H/K polymorphisms within ovine PRNP gene were reported to be associated with susceptibility to the disease.⁸⁻¹² In goat, the M142I/T, N146S/D, H154R and Q222K polymorphisms within the caprine PRNP gene are associated with an extension of the incubation period in an experimental model of classical goat scrapie.¹³⁻²⁰ However, in cattle, none of the known polymorphisms within the PRNP coding region has a major influence on susceptibility to BSE, which is different when compared to other mammals.

Interestingly, in the non-coding region of bovine PRNP gene, Sander et al revealed an association between susceptibility to BSE and a 23 bp insertion/deletion (indel) polymorphism in the promoter and a 12 bp indel polymorphism in intron I using German BSE cases and control cattle.²¹ This observation revealed a novel relationship between susceptibility to classical BSE and specific indel polymorphic sites in two regulatory regions upstream of the coding region of the mammalian PRNP gene. Since 2004, several studies have focused on the associations of promoter polymorphisms within PRNP gene with the susceptibility and incubation time for TSE in bovine. However, no study has focused on investigating the frequency of distribution of two indel polymorphisms within the PRNP promoter region and their association with BSE susceptibility in Chinese dairy cattle population.

It is well known that the main purpose of animal husbandry is the production of milk and meat for human consumption. Thus, animals with more meat and milk are favored to be retained, whereas animals with low productivity are eliminated. This fact can be corroborated by the rapid increase of commercial animal breeds and dramatic decrease of local animal breeds. Cemal et al reported that Turkish local gray cattle were a low productivity breed, but demonstrated the low-susceptibility against BSE-affected.²² Moreover, low BSE susceptibility of gray cattle was significantly associated with rather higher frequencies

of 23ins (0.62) and 12ins (0.80) alleles in the indel mutations within PRNP gene. This observation prompted us to consider the relationship between productivity and allelic frequencies of PRNP gene. To date, we are not aware of any relationship between the 23 bp and 12 bp indel polymorphisms and milk performance traits.

Therefore, the objective of this study is to investigate the 23 bp and 12 bp indel mutations within the bovine PRNP gene in Chinese dairy cattle. Moreover, associations of two indel polymorphisms with BSE susceptibility and performance traits will also be analyzed. These observations will lay the foundation for future selection of resistant animals, and for improving health conditions for dairy breeding against BSE in China.

MATERIALS AND METHODS

Samples and DNA extraction

Blood samples were obtained from 94 healthy and unrelated Chinese Holstein dairy cattle belonging to two different herds. The first herd was comprised of 33 individuals. The second herd has a total of 61 cattle, which were born between 1998 and 2000 and came from 8 fathers. The daughters of 8 sires were 14, 7, 7, 7, 7, 7 and 5 animals, respectively. Cows were fed with a complete ration of artificially dried grass, corn silage and concentrates.²³ The dairy performance records including fat content (Fat %), protein content (Protein %), the total of fat yield (LTDF), the total of protein yield (LTDP), the total of milk yield (LTDM) and 305 d milk yield (M305), and other resistant breast mastitis traits involving somatic cell count (SCC), previous somatic cell count (PreSSC) and linear somatic cell count (LSCC), were obtained during the third lactation. Genomic DNA was isolated from blood samples using a phenol-chloroform method.²⁴

PCR amplification

Based on bovine PRNP gene sequence (GenBank accession No. AJ298878), the first pair of primers [P23F: 5'-GTGCCAGCCATGTAAGTG-3' (nt 47 784 - 47 901) and P23R: 5'-CCTATTCTGGCTATTGTTGC-3' (nt 47 965 - 47 984)], was designed to detect the 23 bp indel polymorphism within bovine PRNP gene (200 bp - 223 bp fragment). The second pair of primers [P12F:

5'-CTCGCAGAAGCAGGTAAATAGCC-3' (nt 49 469 - 49 491) and P12R: 5'-CTAGATTCCTACACACCAC-3' (nt 49 759-49 777)], was designed to detect the 12 bp indel polymorphism with the bovine PRNP gene (308 bp-320 bp fragment). Amplification was done using a BioRad Research PTC200 thermocycler (Hercules, California, USA). The 25 μ l volume contained: 50 ng genomic DNA, 0.5 μ M of each primer, 1 $\times$ Buffer [including 1.5 mM MgCl₂], 200 μ M dNTPs (dATP, dTTP, dCTP and dGTP) and 0.625 units of *Taq* DNA polymerase (MBI, Vilnius, Lithuania). The cycling protocol was 95 °C for 5 min, 35 cycles of 94 °C for 30 s, annealing 56.5 °C for 40 s, 72 °C for 120 s, with a final extension at 72 °C for 10 min.

Detection of the two indel polymorphisms by direct electrophoresis

PCR products were electrophoresed through 10.0% polyacrylamide gel electrophoresis (PAGE) on BioRad (80 $\times$ 73 $\times$ 0.75 mm, USA), in the presence of 1 $\times$ TBE, and fragments detected using autoradiography. Simultaneously, the PCR products were also detected by electrophoresis in 4.0% agarose gel stained with ethidium bromide (EB).

Statistical analysis

Genotype, allele and haplotype frequencies were estimated by counting, based on Lewontin (1988).²⁵ Linkage disequilibrium (D , D' and r^2) was calculated by SHEsis software.²⁶

Based on previous and present genotypic and allelic frequencies of two indels polymorphisms, distribution differences of frequencies between Chinese Holstein and healthy/BSE-affected populations were analyzed using a chi-square test (χ^2 -test). The chi-square test was performed using the software of SPSS V13.0 (SPSS Inc., USA). According to previous reports,²⁷⁻³³ genotypic and allelic frequencies of these indel polymorphisms in healthy dairy cattle from nine dairy cattle populations were collected, namely, Vietnam dairy cattle (VN-dairy, n=206), Japanese Holstein cattle (JPN-HF, n=278), United Kingdom Holstein cattle (UK-HF, n=276), Polish Holstein-Friesian (PLH-HF-F, n=234), AI sires of Polish Holstein-Friesian (PLH-HF-F-AI, n=47), German Holstein I cattle (GER-HF-I, n=313), German Holstein II cattle (GER-HF-II, n=80), Hanwoo dairy (Hanwoo, n=107) and Korean Holstein (Korean-HF,

n=52). Also, genotypic and allelic frequencies of the two indel polymorphisms from twelve BSE-affected cattle populations were collected, namely, Japanese BSE-affected Holstein (JPN-HF-BSE, n=7), Germany BSE-affected cattle (GER-BSE, n=43), United Kingdom BSE-affected Holstein (UK-HF-BSE, n=363), German BSE-affected Holstein group I (GER-HF-BSE-I, n=127), German BSE-affected Holstein group II (GER-HF-BSE-II, n=119), German BSE-affected Brown group I (GER-B-BSE-I, n=43), German BSE-affected Brown group II (GER-B-BSE-II, n=16), German BSE-affected Fleckvieh cattle group I (GER-F-BSE-I, n=106), German BSE-affected Fleckvieh cattle group II (GER-F-BSE-II, n=69), Swiss BSE-affected brown (SWI-B-BSE, n=98), Swiss BSE-affected Schwarzfleck (SWI-S-BSE, n=25) and Swiss Simmental xRed Holstein BSE-affected (SWI-SR-BSE, n=122).

Statistical association analysis was performed on records of performance traits in the second Holstein herd (n=61). The effects associated with season of birth (spring vs. fall), age of dam and sire were not included in the linear model, as the preliminary statistical analyses indicated that these effects did not have significant influence on variability of traits in the population. Therefore, the effects of genotype on the traits were analyzed by the least-squares method as applied in the general linear model (GLM) procedure of SPSS according to the following statistical model: $Y_{ijk} = \mu + G_i + R_j + E_{ijk}$, where, Y_{ijk} is the studied traits; μ is the overall mean; G_i is the fixed effect of the PRNP genotype; R_j is the fixed effect of season at the start of lactating; E_{ijk} is the random residual effect.²³ As the ins/ins genotype is rare (n=1), only two genotypes were entered into the statistical analysis. According to the genotype distribution of two indel polymorphisms, the four combined genotypes (23 bp del/ins-12 bp del/ins, 23 bp del/ins-12 bp del/del, 23 bp del/del-12 bp del/ins, 23 bp del/del-12 bp del/del) were identified. Based on the above statistical model, the effects of these combined genotypes were evaluated except the rare frequencies genotypes.

RESULTS

We detected polymorphic DNA fragments at the 23 bp and 12 bp indel polymorphisms within PRNP gene in Chinese Holstein population. Frequencies of

Figure 1. The electrophoresis pattern of the 23 bp and 12 bp indel loci of bovine PRNP gene.

Notes: Figure 1A revealed the electrophoresis pattern of 23 bp indel locus of bovine PRNP gene; figure 1B revealed the electrophoresis pattern of 12 bp indel locus of bovine PRNP gene. “I” represented “insertion” and “D” represented “Deletion”. Correspondingly, the “II”, “ID” and “DD” represented the “Ins/Ins” genotype, the “Ins/Del” genotype and “Del/Del” genotype, respectively. “M” represented the “marker”. At the 23 bp indel locus, the heterozygous mutation (ID) showed three bands, which might be heteroduplex DNA and did not affect the genotype identification. At the 12 bp indel locus, the heterozygous mutation (ID) show two bands (308 bp and 320 bp).

alleles and genotypes were investigated in analyzed population (Table 1). The observed haplotype frequencies of 23 bp and 12 bp indel polymorphisms in Chinese Holstein populations could be found in Table 2, as well as r^2 , D , D' and χ^2 values can be seen.

The Chi-square tests demonstrated significant distribution differences of genotypic and allelic frequencies in the 23 bp and 12 indel loci from all of the categorized data ($P < 0.001$ and $P < 0.001$, respectively). Compared with the frequencies of the 23 indel locus in several healthy and BSE-affected dairy populations (Table 3), there were significant distribution differences between Chinese Holstein, healthy dairy and BSE-affected cattle ($P < 0.05$ or $P < 0.01$). In the 12 bp indel polymorphism, comparing with the several healthy and BSE-affected dairy populations (Table 4), we found significant distribution differences of genotypic or allelic frequencies among Chinese Holstein, healthy cattle and BSE-affected cattle ($P < 0.05$ or $P < 0.01$).

A test to establish the relationships between the two indel polymorphisms and milk performance traits was performed. However, no statistically significant differences were observed among different genotypes and milk performance traits ($P > 0.05$) (Table 5). Also, the associations of four combined genotypes of 23 bp and 12 bp indel polymorphisms with milk

performance traits were performed in Chinese Holstein populations, but no significant differences were detected ($P > 0.05$) (Table 6).

DISCUSSION

The prion protein hypothesis has suggested that changes in the expression of the endogenous PrP^C might influence susceptibility to TSE and/or TSE incubation time.³⁴ Complying with this hypothesis, the level of PrP^C expression in transgenic mice is inversely correlated with the incubation time for TSE.³⁵ The prion protein disease exhibits an interspecies transmission mechanism, such as from human CJD or vCJD to bovine BSE, from goat scrapie to cattle BSE, from sheep scrapie to cattle BSE.²⁹ Basically, bovine BSE can also be infectious between different cattle populations. Recently, an outbreak of bovine BSE occurred in May 2009 in Canada, which will increase the possibility of spread of BSE disease. Although there is no report about outbreak of bovine BSE in China, care should be taken to avoid infection of Chinese bovine from bovine BSE from other countries or other mammal TSE disease, and selection for resistant cattle could be a good strategy.

Chinese Holstein dairy cow is the most important dairy breed for producing milk related products

Table 1. Genotypic and allelic frequencies of 23 bp and 12 bp indel polymorphisms in Chinese Holstein dairy cows

Indel/Populations (sample number)	Genotypes (%)			Alleles (%)		χ^2 (HWE)	Sample Variation (%)
	del/del	del/ins	ins/ins	del	ins		
23 bp indel polymorphism							
* Holstein (94)	0.48	0.48	0.03	0.73	0.27	4.326	0.11
12 bp indel polymorphism							
* Holstein (94)	0.55	0.44	0.10	0.77	0.23	5.246	0.09

Notes: HWE was Hardy-Weinberg Equilibrium; χ^2 (df = 1) = 3.84, P = 0.05; χ^2 (df = 1) = 6.63, P = 0.01; “*” represented Hardy-Weinberg disequilibrium.

in China. And it is comprised of several different populations, such as Xi'an Holstein population and Beijing Holstein population. However, undertaking the serious case of trimeric cyanamide, the milk related products have been attacked with the decreasing number and production of Chinese Holstein dairy cow. In order to re-open the market of milk related products, the breeding of highly productive and BSE resistant dairy cows has been emphasized by the government of China. Previous researches demonstrated that in bovine, the 23 bp and 12 bp indel polymorphisms within the PRNP gene are associated with susceptibility to BSE. To date, however, no study has related these two indel polymorphisms within the PRNP gene in Chinese dairy cows. There is a gap in the knowledge of the PRNP gene polymorphisms related to BSE susceptibility and the importance of dairy cow for producing milk related products in China, it is essential to analyze the polymorphisms of the PRNP gene and their associations with BSE susceptibility and performance traits in Chinese dairy populations. Therefore, this study has focused on investigating the frequency of distribution of the 23 bp and 12 bp indel polymorphisms within the PRNP gene in Chinese dairy cows, and analyzing their possible associations with BSE susceptibility and performance traits, for the end goal of developing BSE resistant and perfect productive dairy breeds.

We have investigated the two indel polymorphisms in Chinese Holstein population, and found three genotypes and two alleles. The Chinese Holstein population possessing lower “ins” alleles in the 23 bp indel, was at the disequilibrium of Hardy-Weinberg ($P < 0.05$), which may have been related to the strong artificial selection. In the farm of the Chinese Holstein population, the elimination rate of low producing individuals was very high. The same strong artificial selection also occurred in farm of the Chinese Holstein population, which partly explains the popular

heterozygous del/ins of the two indel polymorphisms in the Chinese Holstein population. The 23del-12del and 23ins-12ins were linked at a medium level in the analyzed populations. This observation similarly complied with the previous reported in other populations.^{27,31}

Reporter gene assays have demonstrated that the 23 bp and 12 bp indel polymorphisms are connected with the expression levels of the bovine PRNP

Table 2. haplotype frequencies of 23 bp and 12 bp indel polymorphisms in Chinese Holstein dairy cows

Genotypes	Frequencies
23del-12del/23del-12del	0.353
23del-12del/23del-12ins	0.118
23del-12ins/23del-12ins	0.012
23del-12del/23ins-12del	0.200
23del-12del/23ins-12ins	0.282
23del-12ins/23ins-12ins	0.000
23ins-12del/23ins-12del	0.000
23ins-12del/23ins-12ins	0.035
23ins-12ins/23ins-13ins	0.000
23del-12del	0.653
23del-12ins	0.071
23ins-12del	0.118
23ins-12ins	0.159
23del-12del	0.558
23del-12ins	0.166
23ins-12del	0.213
23ins-12ins	0.063
D value	0.095
D' value	0.575
r^2 value	0.257
χ^2 value	21.875
P value	$P < 0.01$

Table 3. Distribution differences of frequencies at 23 bp indel locus within b PRNP gene among control and BSE cattle populations

Breeds	N	Genotypes (Frequencies, %)	Chinese Holstein		Alleles (del/ins)	Chinese Holstein	Cited
		DD/DI/II	χ^2	Sig.(df = 2)	(Frequencies, %)	Fisher's exact sig.	
VN-dairy	206	70.7/27.8/1.5	14.11	0.001	84.6/15.4	0.001	Muramatsu et al, 2008
JPN-HF	278	63.0/32.0/5.0	8.16	0.017	79.0/21.0	0.085	Nakamitsu et al, 2006
UK-HF	276	46.4/48.9/4.7	0.42	0.812	70.9/29.2	0.708	Juling et al, 2006
PLH-HF-F	234	38.0/50.4/11.6	6.81	0.033	63.2/36.8	0.028	Czarnik et al, 2007
PLH-HF-F-AI	47	38.2/48.9/12.8	5.13	0.077	62.7/37.3	0.101	Czarnik et al, 2007
GER-HF-I	313	38.0/47.3/14.7	9.74	0.008	61.7/38.4	0.007	Juling et al, 2007
GER-HF-II	80	42.5/48.8/8.8	2.60	0.273	66.9/33.2	0.290	Haase et al, 2007
Hanwoo	107	37.4/43.9/18.7	11.98	0.003	59.4/40.7	0.006	Jeong et al, 2006
Korean-HF	52	51.9/36.6/11.5	4.86	0.088	70.2/29.8	0.685	Jeong et al, 2006
JPN-HF-BSE	7	85.7/14.3/0.0	0.35	0.161	92.9/7.15	0.120	Muramatsu et al, 2008
GER-BSE	43	51.0/44.0/5.0	0.18	0.853	73.0/27.0	1.000	Sander et al, 2004
UK-HF-BSE	363	55.4/4.0/13.6/	0.72	0.442	75.9/24.1	0.343	Juling et al, 2006
GER-HF-BSE-I	127	45.7/46.5/7.9	0.13	0.352	69.0/31.2	0.459	Juling et al, 2006
GER-HF-BSE-II	119	42.9/50.4/6.7	0.10	0.443	68.1/31.9	0.337	Haase et al, 2007
GER-B-BSE-I	43	20.9/65.1/14.0	0.00	0.002	53.5/46.6	0.002	Juling et al, 2006
GER-B-BSE-II	16	25.0/56.3/18.8	0.01	0.020	53.2/47.0	0.037	Haase et al, 2007
GER-F-BSE-I	106	53.8/39.6/6.6	0.35	0.321	73.6/26.4	0.822	Juling et al, 2006
GER-F-BSE-II	69	62.3/30.4/7.2	0.19	0.054	77.5/22.4	<0.001	Haase et al, 2007
SWI-B-BSE	98	23.5/35.7/40.7	0.00	<0.001	41.4/58.6	<0.001	Haase et al, 2007
SWI-S-BSE	25	40.0/48.0/12.0	0.07	0.194	64.0/36.0	0.293	Haase et al, 2007
SWI-SR-BSE	122	36.9/51.6/11.5	0.01	<0.001	62.7/37.3	0.038	Haase et al, 2007

Table 4. Distribution differences of frequencies at 12 bp indel locus within bPRNP gene among control and BSE cattle populations

Breeds	N	Genotypes (Frequencies, %)	Chinese Holstein		Alleles (Del/Ins)	Chinese Holstein	Cited
		DD/DI/II	χ^2	Sig. (df = 2)	(Frequencies, %)	Fisher's exat sig.	
VN-dairy	206	14.9/65.6/19.5	59.03	<0.001	47.7/52.3	<0.001	Muramatsu et al, 2008
JPN-HF	278	55.0/38.0/7.0	5.00	0.082	74.0/26.0	0.440	Nakamitsu et al, 2006
UK-HF	276	37.0/51.9/11.1	14.80	0.001	63.0/37.1	<0.001	Juling et al, 2006
PLH-HF-F	234	0.03/49.0/21.0	29.45	<0.001	55.0/46.0	<0.001	Czarnik et al, 2007
PLH-HF-F-AI	47	19.1/63.9/17.0	24.52	<0.001	51.1/49.0	<0.001	Czarnik et al, 2007
GER-HF-I	313	28.2/49.8/22	34.46	<0.001	53.1/46.9	<0.001	Juling et al, 2007
GER-HF-II	80	33.8/55.0/11.3	13.38	0.001	61.3/38.8	0.002	Haase et al, 2007
Hanwoo	107	31.8/48.6/19.6	22.50	<0.001	56.1/43.9	<0.001	Jeong et al, 2006
Korean-HF	52	38.6/41.4/20.0	16.61	<0.001	59.3/40.7	0.002	Jeong et al, 2006
JPN-HF-BSE	7	85.7/14.3/0.0	2.47	0.291	92.9/7.15	0.311	Muramatsu et al, 2008
GER-BSE	43	44.0/47.0/9.0	22.48	<0.001	67.5/32.5	<0.001	Sander et al, 2004
UK-HF-BSE	363	49.4/45.4/5.1	3.44	0.179	72.1/27.8	0.194	Juling et al, 2006
GER-HF-BSE-I	127	40.0/45.6/14.4	13.75	0.001	62.8/37.2	0.002	Juling et al, 2006
GER-HF-BSE-II	119	34.5/52.9/12.6	15.48	<0.001	61/39.1	<0.001	Haase et al, 2007
GER-B-BSE-I	43	7.0/51.2/41.9	52.95	<0.001	32.6/67.5	<0.001	Juling et al, 2006
GER-B-BSE-II	16	6.3/43.8/50.0	46.85	<0.001	28.2/71.9	<0.001	Haase et al, 2007
GER-F-BSE-I	106	45.3/46.2/8.5	6.58	0.037	68.4/31.6	0.057	Juling et al, 2006
GER-F-BSE-II	69	52.2/39.1/8.7	5.66	0.059	71.8/28.3	0.302	Haase et al, 2007
SWI-B-BSE	98	7.1/37.8/55.1	85.55	<0.001	26.0/74.0	<0.001	Haase et al, 2007
SWI-S-BSE	25	28.0/56.0/16.0	14.11	0.001	56.0/44.0	0.004	Haase et al, 2007
SWI-SR-BSE	122	23.8/49.2/27.0	37.22	<0.001	48.4/51.6	<0.001	Haase et al, 2007

Table 5. Associations of different genotypes of 23 bp or 12 bp indel polymorphisms with performance traits in Holstein population (Mean $\pm$ SD)

Traits	23 bp indel locus			12 bp indel locus		
	de/del	del/ins	<i>P</i> value	de/del	del/ins	<i>P</i> values
	0.60 (Frequencies)	0.38 (Frequencies)		0.51 (Frequencies)	0.47 (Frequencies)	
305 d milk yield (M305) (kg)	6894 $\pm$ 198	6299 $\pm$ 2356	0.420	6182 $\pm$ 2074	7555 $\pm$ 2178	0.082
Total of milk yield (LTDM) (kg)	5035 $\pm$ 2351	5267 $\pm$ 2418	0.218	4776 $\pm$ 2301	5358 $\pm$ 2423	0.413
Total of protein yield (LTDP) (kg)	155 $\pm$ 83	144 $\pm$ 68.50	0.283	135 $\pm$ 69.60	162 $\pm$ 80.50	0.225
Total of fat yield (LTDF) (kg)	180 $\pm$ 87.70	188 $\pm$ 1050	0.328	171 $\pm$ 96.80	194 $\pm$ 90.30	0.427
Fat %	3.67 $\pm$ 0.69	3.65 $\pm$ 0.60	0.779	3.73 $\pm$ 0.70	3.69 $\pm$ 0.35	0.825
Protein %	3.00 $\pm$ 0.20	3.10 $\pm$ 0.24	0.800	3.03 $\pm$ 0.24	3.10 $\pm$ 0.21	0.324
Fat content /Protein content (F/P)	1.20 $\pm$ 0.14	1.19 $\pm$ 0.18	0.960	1.21 $\pm$ 0.12	1.19 $\pm$ 0.12	0.653
Somatic cell count (SCC)	717 $\pm$ 747	616 $\pm$ 710	0.680	705 $\pm$ 805	649 $\pm$ 671	0.807
Previous somatic cell count (PreSSC)	783 $\pm$ 845	500 $\pm$ 436	0.219	754 $\pm$ 885	595 $\pm$ 388	0.528
Linear somatic cell count (LSCC)	4.21 $\pm$ 1.45	4.01 $\pm$ 1.14	0.073	4.00 $\pm$ 1.62	4.20 $\pm$ 1.13	0.667

Table 6. Associations of combined genotypes of two indels polymorphisms with performance traits in Holstein population (Mean $\pm$ SD)

Traits	Combined genotypes of 23 bp and 12 bp indel loci				<i>P</i> values
	23del/ins	23del/ins	23del/del	23del/del	
	12del/ins	12 del/del	12 del/ins	12del/del	
	0.26 (Frequencies)	0.16 (Frequencies)	0.13 (Frequencies)	0.42 (Frequencies)	
305d milk yield (M305) (kg)	7147 $\pm$ 2064	5249 $\pm$ 1663	8369 $\pm$ 487	6658 $\pm$ 2012	0.162
Total of milk yield (LTDM) (kg)	5441 $\pm$ 2015	4234 $\pm$ 2158	6066 $\pm$ 1697	5293 $\pm$ 1845	0.543
Total of protein yield (LTDP) (kg)	152 $\pm$ 56.40	120 $\pm$ 48.7	180 $\pm$ 61.10	149 $\pm$ 50.70	0.428
Total of fat yield (LTDF) (kg)	191 $\pm$ 68.30	142 $\pm$ 62.4	224 $\pm$ 59.50	194 $\pm$ 78.30	0.379
Fat %	3.80 $\pm$ 0.24	3.71 $\pm$ 0.41	3.58 $\pm$ 0.22	3.60 $\pm$ 0.33	0.514
Protein %	3.07 $\pm$ 0.16	2.98 $\pm$ 0.09	2.98 $\pm$ 0.13	3.00 $\pm$ 0.24	0.800
Fat content /Protein content (F/P)	1.26 $\pm$ 0.09	1.25 $\pm$ 0.13	1.21 $\pm$ 0.10	1.22 $\pm$ 0.11	0.780
Somatic cell count (SCC)	481 $\pm$ 337	223 $\pm$ 131	689 $\pm$ 354	757 $\pm$ 953	0.486
Previous somatic cell count (PreSSC)	499 $\pm$ 349	265 $\pm$ 201	759 $\pm$ 481	812 $\pm$ 1084	0.554
Linear somatic cell count (LSCC)	4.07 $\pm$ 0.78	3.20 $\pm$ 0.71	4.50 $\pm$ 0.95	3.80 $\pm$ 1.69	0.483

gene.^{36,32} The 23 bp and 12 bp indel polymorphic sites in the PRNP promoter contain binding sites for RP58 and SP1 transcription factors, respectively. Their function analysis revealed an interaction between the two transcription factors and lower PRNP expression levels of the allele harboring “ins” in the 23ins-12ins site compared with the other allele harboring “del” in the 23del-12del site.³⁶ The effect of the 12 bp indel polymorphism was recently confirmed in independent studies using German and UK cattle.^{27,32} Also, a haplotype consisting of 12 bp indel polymorphisms and SNPs is associated with the lowest expression level and has been shown to correlated with reduced PRNP expression and increased resistance to BSE.³²

These observations upheld that mutations potentially influencing the level of bovine prion protein expression might also influence the incubation time and susceptibility to BSE.

Since 2004, a few studies have focused on the associations of the indel polymorphisms within the PRNP gene with the susceptibility and incubation time for TSE in bovine. Among the polymorphisms, three comments on influences of the two indel polymorphisms on bovine BSE have been found: positive influence, negative influence and breed dependent influence. First comment: two indel polymorphisms have been shown to have positive influence on BSE. A 23 bp in the putative promoter

region and a 12 bp within intron I, had been founded to associate with resistance to prion infection in German cattle, both insertions being more frequent in the analyzed animals against BSE.^{21,27,32,27} Second comment: two indel polymorphisms have also been shown to have negative influence on BSE in their study. In this study, the effect of the 12 bp indel on BSE susceptibility was not found in 252 BSE animals and 376 controls, which complied with the findings of Nakamitsu et al,²⁸ Czarnik et al³⁰ and Haase et al.³¹ Third comment: breed dependent influence of the two indels on BSE has also been demonstrated by several groups. Brunelle et al³⁸ described that indel polymorphisms that influence classical BSE susceptibility do not seem to be applicable to other transmissible spongiform encephalopathies in cattle, which was identical with the findings of Kerber et al³⁹ and Cemal et al.²² In Vietnamese dairy cattle, Muramatsu et al.³³ described that the frequency distributions of del allele and del/del genotypic polymorphisms in the 23 indel site, which are thought to be associated with BSE susceptibility, were significantly higher, whereas the frequencies of del allelic and del/del genotypic polymorphisms in the 12 indel site, which have been reported to confer BSE susceptibility, were significantly lower.

According to the Chi-square test of this study, we found significant distribution differences of genotypic and allelic frequencies at 23 bp and 12 bp indel polymorphisms among Chinese Holstein populations and some healthy dairy populations from previously reported data. This finding suggested that Holstein populations from different countries have an effect on the frequencies of distribution of two indel polymorphism due to their different genetic backgrounds. For example, Muramatsu et al³³ described that the frequency distributions of the “del” allele and the “del/del” genotypic polymorphisms in two indel sites in Vietnam dairy cows was due to their specific genetic background. Herein, significant distribution differences of genotypic and allelic frequencies were also found between HN&XA Holstein population and Vietnam dairy cow, which confirmed the special genetic background of the Vietnam dairy cow. In addition, we also found significant distribution differences of genotypic and allelic frequencies of the two indel polymorphisms among the Chinese Holstein population and some BSE-affected populations from the previous data

of the infectious regions. This seems to imply that the BSE susceptibility is significantly associated with the frequencies of distribution of two indel polymorphisms. The 23 bp del and 12 bp del alleles had a significant effect on the high-susceptibility of BSE disease in some breeds, but not in all breeds. This study supported the idea that the two indels influence BSE in a breed dependent manner. The main explanation of this finding is the fact that each breed has a different genetic background which affects their susceptibility to BSE disease.

The association analysis results revealed no significant effects of PRNP gene on performance traits of Chinese Holstein, which was in accordance with similar reports in sheep.⁴⁰ In the V136A, R154H, Q171R/H/K polymorphisms within the bovine PRNP gene, no effects of the sire genotype (ARR/ARR and ARR/ARQ) were found for any of the traits in Chios sheep. Moreover, the 60-day milk yield after weaning was not influenced by the genotype (ARR/ARR, ARR/ARQ and ARQ/ARQ genotypes on 136, 154 and 171 loci, respectively). These observations suggested that the two indel polymorphisms are neutral mutation regions, which do not affect the production traits. Therefore, the production traits are not important when considering the BSE resistance capabilities in dairy cattle.

To summarize, the present work investigated the frequencies of distribution of the 23 bp and 12 bp indel polymorphisms of Chinese Holstein dairy cows, and revealed that the two indel loci did not affect performance traits. Moreover, this study supported the hypothesis that the influence of two indel polymorphisms on BSE was breed dependent.

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