

ORIGINAL ARTICLE

Detection of Haplotype and Polymorphisms of the Prion Protein Gene in Holstein and Three Iranian Native Cattle Breeds

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ABSTRACT

Transmissible spongiform encephalopathies are neurodegenerative diseases that affect several mammalian species. Bovine spongiform encephalopathy (BSE) is among the mentioned diseases in cattle caused by abnormally fold prion proteins. Polymorphism (PRNP) as 23-bp and 12-bp insertion and deletion in promoter and intron 1, in both loci are related to resistance but deletion alleles affect inversely. We studied allele, genotype and haplotype frequencies of these two loci among samples of two Iranian cattle breeds including Najdi, Sarabi and Iranian Holstein. Polymorphisms of the 23-bp indel in the promoter region and the 12-bp indel in intron 1 of the prion protein gene, which are shown to be associated with resistance to bovine spongiform encephalopathy (BSE), were assessed in the imported Holstein (n=55) and native Iranian Sarabi (n=56) and Najdi (n=55) cattle breeds. Najdi is a *B. indicus* breed of Southern Iran and Sabari is a *B. taurus* breed of north-western part of the country. Holstein breed had significantly different allele, genotype and haplotype frequency distributions compared with the two native breeds for both polymorphic sites, except for the 23-bp indel which was comparable with the Sarabi breed. The frequencies of the protective 23-bp insertion allele (23+) were 0.28, 0.15 and 0.33 in the Sarabi, Najdi and Holstein breeds, respectively. The corresponding values for the low susceptibility 12-bp insertion allele (12+) were 0.92, 0.89 and 0.48, respectively. The frequencies of this allele in the two native breeds are at the upper limit of most breeds studied so far. The allele and haplotype frequency distributions of the Najdi breed resembled that of the published data on other *B. indicus* cattle. It is not possible to predict the degree of susceptibility of the native breeds to BSE based on the frequencies of these two polymorphic sites because of uncertainties regarding the relative contribution of each site to the overall resistance to BSE and the presence of breed-specific background genes affecting this trait.

Keywords; Polymorphisms. Prion protein gene (PRNP). Genetic susceptibility, Genetic resistance, Native cattle breeds

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INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a serious animal health issue as well as a human food concern because it is transmittable to humans [1, 2]. BSE is particularly important in countries such as Iran where the cattle brain often enters the human food chain. Protecting the national cattle population against BSE is not only essential for the country's livestock industry, but also for the human health. The presence of BSE has not been investigated in Iran, and there is no information on the degree of susceptibility of the indigenous cattle breeds to BSE infection.

Recent reports on the significant associations between the 23-bp insertion/deletion (indel) polymorphism in the promoter region and the 12-bp indel within intron 1 of the bovine prion protein gene (PRNP) with BSE resistance in cattle breeds of Germany, Switzerland and Britain [3-7] generated a considerable interest among scientists globally. Although differences between healthy and infected cattle for allele, haplotype and/or genotype frequencies were statistically significant in those studies, and animals carrying the insertion alleles at both sites (23+ and 12+) were more resistant to BSE, there is no agreement on the relative influence of each allele on resistance to BSE infection. The favourable alleles decrease the odds of the establishment of infection when an animal is exposed to the BSE agent, but do

not confer complete protection as indicated by the presence of all genotypes in both healthy and infected cattle [3,5, 6]. Associations between these two polymorphisms and the incidence of BSE were not detected for the German and Swiss Brown breed [7, 4] or the Holstein breed in Japan [8]. Furthermore, the association between these polymorphisms and infection by non-classical BSE agents has not been established yet [9].

The picture that emerges from the published information on the allele frequency distributions of various breeds is, nevertheless, interesting. The *B. indicus* cattle have significantly higher frequencies of the 23- and the 12+ alleles as compared to *B. taurus*, resulting in the 23-/12+ to be the major haplotype in *B. indicus* [10].

Significant differences among cattle breeds for allele and haplotype frequencies may arise because of i-founder effect and random genetic drift, particularly if the population has gone through a bottleneck. Such phenomenon should cause parallel changes in many loci. ii-natural selection for resistance to BSE, which requires that BSE has been epidemic in the region, and iii-linkage with other genes that influence production performance or adaptability to certain environmental conditions. Genotyping of cattle breeds across the globe with different breeding histories and production performances may shed some light on the relative importance of this gene on BSE resistance.

The objective of this study was to determine the allele, genotype and haplotype frequencies of two indigenous cattle breeds of Iran; Sarabi and Najdi. A sample of Holstein cattle was used for comparison. The information is useful in assessing the natural selection pressure imposed on this gene as well as the relative level of resistance of indigenous breeds to classical BSE.

MATERIALS AND METHODS

Breeds: Najdi is a *Bos indicus* breed of Iran whose origin is not clearly known. The majority of estimated 380,000 heads of the indigenous cattle of the Khozestan Province in South-western Iran comprised of the Najdi breed with the average daily milk yield of 6-8 kg during 125-day lactation period with 5.8% milk fat (Khosestan Agricultural research Center, 2006). This breed is adapted to the high temperatures of the region, which often reached 50°C in the summer.

Sarbi is the most famous indigenous cattle breed, which is mainly distributed in north-western part of Iran (Azarbayjan Province). Daily milk production of this breed ranges between 6 and 14 litres during 250 days lactation period with 5.4% milk fat (<http://www.skhass.org/site/?itemid=541>). Sarabi is also adapted to local conditions, and is particularly known to be resistant to ticks.

The Holstein breed was imported to Iran in late 1950, and has become the most popular exotic dairy breed of the country. The breed comprises approximately 10% of the 7.9 million cattle population of Iran. Artificial insemination with semen from North America and Europe is a common practice.

Source of animals: Blood samples were collected into 3mL EDTA-treated vacutainers from 55 Holstein, 56 Sarabi and 55 Najdi cattle breeds. Samples of the Sarabi breed were collected from Azarbayjan Agricultural Research Centers in Ardabil (16 of 597 heads), Sarab (15 of 580 heads) and Moghan (25 of 650 heads). Samples of the Najdi breed were collected from Khozestan Agricultural Research Centers in Ahvaz (26 of 675 head), Shooshtar (15 of 598 heads) and Hendijan (14 of 527 heads). Samples of the Holstein breed were collected from Research Stations in Yaasouj (21 of 909 heads), Karaj (18 of 781 heads) and Kermanshah (16 of 526 heads), which are located in different provinces. Animals were randomly selected for sampling.

Laboratory procedures: Genomic DNA was extracted from whole blood using the salting-out procedure of Miller *et al.* [11]. Two regions of the bovine PRNP gene, namely the 12-bp indel in intron 1 and the 23-bp indel in the promoter were amplified by the polymerase chain reaction (PCR). The 100 or 123-bp fragment containing the 23-bp indel was amplified by the primers PRNP47784-F (5'-GTGCCAGCCATGTAAGTG) and PRNP47883-R (5' TGGACAGGCACAATGGG), and the 91 or 103-bp fragment containing the 12-bp indel was amplified by the primers PRNP49686-F (5' TTACCCTCCTGGTTAGGAG) and PRNP49777-R (5' CTAGATTCCTACACACCAC) that were described by Sander *et al.* [3].

PCR was carried out in a 25 µl reaction volume containing 50-100 ng DNA, 1 unit Taq polymerase (Gen Net Bio, Tehran), 20 pM of each primer, 5 mM dNTPs (Gen Net Bio) and 2.5 µl 10X PCR buffer supplied by the manufacturer and contained 1.5 mM MgCl₂. The thermal cycler (Corbett Life Sciences, Australia) was programmed for 5 min initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 90 s, with a final extension step at 72°C for 5 min. The PCR products were size differentiated on 8% polyacrylamide gels and fragments were visualized by silver nitrate.

Allele and genotype frequencies were determined by direct count. Haplotypes were subjectively inferred from genotype information using the fact that homozygous individuals carry a single haplotype, those heterozygous at a single site carry two haplotypes [12], and the haplotype 23+/12- is very rare in cattle.

Statistical Analysis

Differences among breeds for allele frequencies were tested by the Fisher's exact test, and genotype and haplotype frequency distributions were compared with Chi-square using the SAS statistical software (2005). One rare 23+/12- haplotype was detected in the Holstein breed and was not included in the analysis.

RESULTS

Results of this study as follows: Allele and genotype frequencies for the 23-bp and 12-bp in/del regions were compared between two Iranian native cattle and Iranian Holstein presented in Table 1 and 2 respectively. As the shown in table 1, in the 23-bp in/del polymorphism of the PRNP promoter, the frequency of the deletion (-) allele in Najdi breed was higher than the deletion (-) allele in Iranian Holstein and Sarabi breeds. Iranian native cattle possesses a significantly difference in the allele ($P=0.012$) but not the genotype ($P=0.045$) distribution of 23-bp in/del polymorphism was observed between Najdi and Sarabi breeds. The frequencies of the protective 23+ (0.28) and 12+ alleles (0.92) in the Sarabi breed were higher than those in the Najdi breed, 0.15 and 0.89, respectively. Haplotype frequencies where as the most frequent haplotype in Iranian native cattle were 23del-12ins presented in Table 3. The most frequent haplotype in Iranian Holstein cattle was 23del-12del and after that 23ins-12ins, where as the most frequent haplotype in Iranian native cattle was 23del-12ins. Haplotype 23ins-12del occurs in <1% in all populations and was, therefore, excluded from further analyses. The Sarabi breed had a higher frequency of the 23+/12+ haplotype and a lower frequency of the 23-/12- haplotype compared with the Najdi breed. All comparisons involving Holstein and Najdi breeds were highly significant. Holstein had greater values compared with Najdi for the 23+ and 12- alleles and the 23+/12+ and 23-/12- haplotypes. Holstein and Sarabi had comparable allele and genotype frequencies for the 23-bp in/del, but Sarabi had a greater frequency of the 12+ allele, a lower frequency of the 23-/12- haplotype and a greater frequency of the 23-/12+ haplotype than the Holstein breed. Differences between the two native breeds were significant ($P<0.05$) or approached significance ($P=0.053$) for allele, genotype and haplotype frequency distributions. Differences among the breeds for haplotype frequencies were highly significant. While the 23-/12+ was the most frequent haplotype in the two native breeds (0.62 for Sarabi and 0.72 for Najdi), it had the lowest frequency in the Holstein breed. In contrast, the 23-/12- haplotype had the highest frequency in Holstein and the lowest frequency in the native breeds.

Table 1. Comparison of allele frequencies in three Iranian cattle breeds

Breed	Locus	Allele Frequencies			P-Value	
		No	Ins	del	Sarabi	Najdi
IR Holstein	23-bp	55	0.33	0.48	0.311	0.000
Sarabi	23-bp	56	0.28	0.92	-	0.012
Najd	23-bp	55	0.15	0.89	0.012	-
	12-bp	No	Ins	del	Sarabi	Najdi
IR Holstein	12-bp	55	0.33	0.48	0.311	0.000
Sarabi	12-bp	56	0.28	0.92	-	0.053
Najdi	12-bp	55	0.15	0.89	0.053	-

Table 2. Comparison of Genotypic in three Iranian cattle breeds

Breed	Locus	Genotype Frequencies				P-Value	
		No	ins/ins	ins/del	del/del	Najd	Holstein
Holstein	23-bp	55	0.14	0.38	0.48	0.000	-
Sarabi	23-bp	56	0.07	0.42	0.51	0.045	0.511
Najd	23-bp	55	0.05	0.20	0.75	-	0.008
	12-bp	No	ins/ins	ins/del	del/del	Najd	Holstein
Holstein	12-bp	55	0.30	0.36	0.34	0.000	-
Sarabi	12-bp	56	0.87	0.10	0.03	0.008	0.000
Najdi	12-bp	55	0.81	0.16	0.03	-	0.000

Table 3. Comparison of haplotype in three Iranian cattle breeds

Breed	Haplotype	Haplotype Frequencies				P-Value	
		No	ins/ins	del/ins	del/del	Sarabi	Najdi
Holstein	23bp/12bp	55	0.33	0.12	0.55	0.000	0.000
Sarabi	23bp/12bp	56	0.29	0.62	0.09	-	0.008
Najdi	23bp/12bp	55	0.15	0.72	0.13	0.008	-

Genotype and allele frequencies of the 23-bp and 12-bp indels in Holstein and the native Sarabi and Najdi breeds are shown in Tables 4 and 5. Differences between the two native breeds were significant ($P < 0.05$) or approached significance ($P = 0.053$) for allele, genotype and haplotype frequency distributions. The frequencies of the protective 23+ (0.28) and 12+ alleles (0.92) in the Sarabi breed were higher than those in the Najdi breed (0.15 and 0.89, respectively). The Sarabi breed had a higher frequency of the 23+/12+ haplotype and a lower frequency of the 23-/12- haplotype compared with the Najdi breed (Table 5).

Table 4. Genotype frequencies for the 23-bp and 12-bp insertion/deletion polymorphism in Sarabi, Najdi and Holstein breeds

Locus	Breed	n	Frequencies			P-values	
			++	+-	--	Najdi	Holstein
23-bp	Sarabi	56	0.07	0.42	0.51	0.045	0.511
	Najdi	55	0.05	0.20	0.75	-	0.008
	Holstein	55	0.14	0.38	0.48		-
12-bp	Sarabi	56	0.87	0.10	0.03	0.008	0.000
	Najdi	55	0.81	0.16	0.03	-	0.000
	Holstein	55	0.30	0.36	0.34		-

Table 5. Frequencies for the 23-bp and 12-bp insertion alleles in Sarabi, Najdi and Holstein breeds

Locus	Breed	Frequency of + allele	P-values	
			Najdi	Holstein
23-bp	Sarabi	0.28	0.012	0.311
	Najdi	0.15	-	0.003
	Holstein	0.33		-
12-bp	Sarabi	0.92	0.053	0.000
	Najdi	0.89	-	0.000
	Holstein	0.48		-

All comparisons involving Holstein and Najdi breeds were highly significant. Holstein had greater values compared with Najdi for the 23+ and 12- alleles and the 23+/12+ and 23-/12- haplotypes. Holstein and Sarabi had comparable allele and genotype frequencies for the 23-bp indel, but Sarabi had a greater frequency of the 12+ allele, a lower frequency of the 23-/12- haplotype and a greater frequency of the 23-/12+ haplotype than the Holstein breed.

Differences among the breeds for haplotype frequencies were highly significant (Table 6). While the 23-/12+ was the most frequent haplotype in the two native breeds (0.62 for Sarabi and 0.72 for Najdi), it had the lowest frequency in the Holstein breed. In contrast, the 23-/12- haplotype had the highest frequency in Holstein and the lowest frequency in the native breeds.

Table 6. Haplotype frequencies for the 23-bp and 12-bp insertion/deletion Polymorphism in Sarabi, Najdi and Holstein breeds

Breed	Frequencies			P-values	
	23+/12+	23-/12+	23-/12-	Najdi	Holstein
Sarabi	0.29	0.62	0.09	0.008	0.000
Najdi	0.15	0.72	0.13	-	0.000
Holstein	0.33	0.12	0.55		-

Number of animals is shown in Table 1

DNA amplification and Gel doc figure

The PCR products were separated on exact DNA and 8% polyacrylamide gel as shown in figure 1 and 2 respectively.

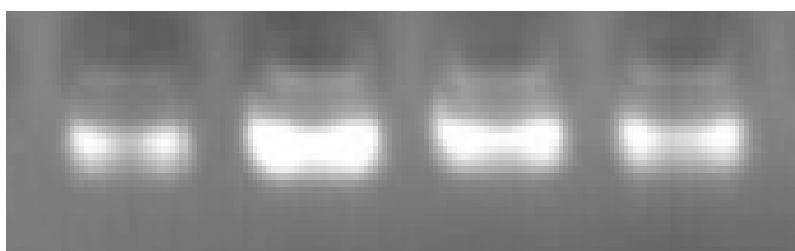


Figure 1. The PCR products DNA amplification



Figure 2. The PCR products were separated on 8% polyacrylamid gel

DISCUSSION

Significant differences were detected among the breeds for the frequencies of the two polymorphisms associated with differential susceptibility to BSE. Allele frequencies for the 23+ and 12+ alleles and the haplotypes for the Holstein breed were comparable with the published information on this breed.

The observation that the 12+ allele was present in a high frequency in the native Sarabi breed deserves attention. First, this breed may possess an important genetic merit for selection for resistance for BSE, particularly because this polymorphism is shown to be the major determinant of resistant against BSE infection, at least in some breeds.

Second, the 12+ has a high frequency in several other unimproved breeds, such as Franqueiro, which is an unimproved old breed adapted to unfavorable environmental conditions of southern Brazil [13]. Korean Hanwoo [14] and three Anatolian cattle breeds [15].

This phenomenon is too common in unimproved breeds to be attributed to a bottleneck or founder effect, as suggested by Kerber *et al* [13]. It may be hypothesized that 12+ is the ancestral allele, and intense selection for production traits has resulted in the reduced frequency of this allele. Alternatively, natural selection under harsh environmental condition may favour this allele.

There is certainly a need for a national BSE monitoring program and tight import regulations to protect the native breeds against the BSE agents.

Until now, it is unknown which polymorphism of bovine PRNP region (23-bp promoter or 12-bp intron 1), may be more important with respect to differential resistance to classical BSE. Either the 23-bp promoter region or the 12-bp intron 1 region of the bovine PRNP are likely to prove more biologically relevant to the incidence of BSE disease. Brunell *et al.* [16] indicated the *Bos indicus* purebred cattle had a very low frequency of the 23-bp insertion and a high frequency of the 12-bp insertion in contrast with *Bos taurus*. Also 23del-12ins was the most frequent haplotype in *Bos indicus*. The current study supports these results especially concerning Najdi cattle as a *Bos indicus* population. However, allele frequencies of 23-bp locus in Sarabi breed was not significantly different from that of Iranian Holstein. Significant differences among cattle breeds for allele and haplotype frequencies may arise because of I-founder effect and random genetic drift, particularly if the population has gone through a bottleneck. Such phenomenon should cause parallel changes in many loci. II - natural selection for resistance to BSE, which requires that BSE has been epidemic in the region, and iii-linkage with other genes that influence production performance or adaptability to certain environmental conditions. Genotyping of cattle breeds across the globe with different breeding histories and production performances may shed some light on the relative importance of this gene on BSE resistance. From the point of frequency of region 23-bp in/del, Sarabi cattle have no significant difference with Holstein breed. However, insertion of region 23-bp in Sarabi breed was significantly higher than Najdi breed. From the point of frequency of region 12-bp in/del, Sarabi cattle were not significant different from Najdi breed, but Sarabi breed were significantly higher than Iranian Holstein breed for insertion of 12-bp region. If only these both mentioned regions affect

resistance to BSE, Sarabi breed have potentially more resistance to BSE, compare to Najdi and Iranian Holstein breed.

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Appendix

	N	23 bp +	12 bp +	23+ / 12+	23+ / 12-	23- / 12+	23- / 12-		
Sarabi	56	0.29	0.92	0.29		0.62	0.09	Iran	Present work
Najdi	55	0.15	0.89	0.15		0.72	0.13		
Holstein	55	0.33	0.48	0.33		0.12	0.55		
Holstein- Total	1380	0.43	0.47	0.43		0.04	0.52	US	[9]
Holstein- Lowest values	88	0.31						Minnesota	
	104		0.38					Tennessee	
	88			0.31				Minnesota	

	120					0.0		California	
	104						0.46	Tennessee	
Holstein, highest values	120	0.55	0.55					California	
	120			0.55				California	
	192					0.08		Minnesota	
	152						0.62	Texas	
Holstein-sires of 1950s	10	0.35	0.55	0.35		0.20	0.45		

Healthy animals only

	N	23 bp +	12 bp +	23+/ 12+	23+/ 12-	23-/12+	23-/ 12-		
Sarabi	56	0.29	0.92	0.29		0.62	0.09	Iran	Present work
Najdi	55	0.15	0.89	0.15		0.72	0.13		
Holstein	55	0.33	0.48	0.33		0.12	0.55		
South Anatolian Red	50	0.36	0.69	0.295	0	0.46	0.245	Turkey	[15]
East Anatolian Red	50	0.40	0.72	0.335	0	0.47	0.195		
Anatolian Gray	50	0.62	0.80	0.62	0	0.21	0.18		
7 cattle breeds *	48	0.43	0.49	-	-	-	-	Germany	[3]
Holstein *	276	0.29	0.37	0.29	<1%	0.08	0.63	UK	[5]
Holstein *	335	0.38	0.47	0.39	<1%	0.09	0.51	Germany	
Brown *	87	0.65	0.86	0.66	<1%	0.19	0.14		
Fleckvieh *	136	0.32	0.38	0.33	<1%	0.05	0.62		
Holstein	278	0.21	0.26	-	-	-	-	Japan	[8]
Japanese Black	186	0.41	0.43	-	-	-	-	"	
Hanwoo	107	0.41	0.44	0.40	0	0.04	0.55	Korea	[14]
Holstein	52	0.30	0.39	0.30	0.01	0.09	0.62	"	
Holstein		0.42	0.47					Germany	[6]
Braunvieh		0.60	0.84						
Fleckvieh		0.29	0.36						
Brown *	206	0.61	0.74	0.61		0.17	0.22	Swiss	[7]
Schwafleck *	52	0.52	0.58	0.52		0.06	0.42	"	
Simmental x Red Holstein *	242	0.45	0.53	0.45		0.08	0.47	"	
Brown *	82	0.56	0.87	0.56		0.28	0.16	Germany	
Fleckvieh *	120	0.31	0.57	0.31		0.07	0.63	"	
Holstein *	160	0.33	0.39	0.33		0.06	0.61	"	
B. indicus	116	0.12	0.87	0.12		0.75	0.13	Many breeds in each	[9]
Composite	76	0.20	0.47	0.20		0.28	0.53		
B. taurus	455	0.38	0.45	0.40		0.08	0.53		
Aberdeen Angus	99	0.27	0.44	0.25	0.02	0.19	0.54	Brazil	[13]
Charolais	82	0.32	0.42	0.31	0.01	0.11	0.57		
Franqueiro	73	0.36	0.67	0.36	0	0.31	0.33		

* Healthy animals only. Franqueiro (Brazil): Unimproved local breed, first cattle brought to southern Brazil by Portuguese in 16th century. Highly adapted to environmental conditions. Also called Brazilian Creole.

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