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RESEARCH ARTICLE

Deletion/insertion polymorphisms of the prion protein gene (*PRNP*) in gayal (*Bos frontalis*)

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Abstract. Resistance to fatal disease bovine spongiform encephalopathy (BSE), due to misfolded prion protein in cattle, is associated with a 23-bp indel polymorphism in the putative promoter and a 12-bp indel in intron 1 of the *PRNP* gene. Gayal (*Bos frontalis*) is an important semiwild bovid species and of great conservation concern, but till today these indel polymorphisms have not been evaluated in gayals. Therefore, we collected 225 samples of gayals and evaluated the genetic indel polymorphism in the two regions of this *PRNP* gene. The results revealed high allelic frequencies of insertions at these indel sites: 0.909 and 0.667 for, respectively, the 23 bp and 12 bp indels, both also with significant genotype frequencies (χ^2: 9.81; 23 bp and χ^2: 43.56; 12 bp). At the same time, the haplotype data showed indel polymorphisms with extremely low deletion (0.01) in both regions of the *PRNP* gene. We compared these data with those reported for healthy and BSE-affected cattle (*Bos taurus*) breeds from two European countries, Germany and Switzerland, and significant difference ($P < 0.001$) was observed between BSE-affected as well as the healthy cattle. Further, our data were also extensively compared with previous reports on BSE and highly significant ($P < 0.001$) outcomes were observed. This result suggested negligible genetic susceptibility to BSE in gayals. To the best of our knowledge, this study is the first comprehensive deciphering information about the *PRNP* indel polymorphisms of 23 bp and 12 bp in gayals, a semiwild species of China.

Keywords. gayal; bovine spongiform encephalopathy; *PRNP* gene; indels.

Introduction

Bovine spongiform encephalopathy (BSE) diagnosed both clinically and pathologically as a neurodegenerative disorder that seriously affects the health of cattle. Its clinical features include aggressive behaviour and ataxia, while the pathological features are prion protein misfolding with aggregation followed by brain's spongiform degeneration (Imran *et al.* 2012). Prion protein plays a fundamental role in the pathogenesis of transmissible spongiform encephalopathies (TSEs). This protein is encoded by the prion protein gene (*PRNP*) which is a polymorphic gene associated with influencing BSE vulnerability in cattle. Among these, a number of polymorphisms and octapeptide repeats in coding sequence and two insertion/deletion (indel) polymorphisms characterized by a 23-bp indel in the putative promoter and a 12-bp indel in intron 1 were retrieved (Zhao *et al.* 2015). Previous studies revealed that these two traits emanated from the deletion of alleles either at a single or both 23 bp and 12 bp indel sites, associated with higher susceptibility to classical BSE (Sander *et al.* 2004; Juling *et al.* 2006). However, BSE has not been reported in gayal (Xi *et al.* 2011), a semiwild endangered bovine species with no defined background (Mei *et al.* 2016). The reports of gayal from Bangladesh, Bhutan, China, India and Myanmar have confirmed its possession of a different chromosome complement of $2n = 58$ (while cattle $2n = 60$ and gaur $2n = 56$; Xi *et al.* 2012).

There are no evidence of successful cross-breeding between gayal and other close relatives, thus the species still remains natural to date with morphological features such as bulging forehead ridge, wide and short ears and feet

with white stockings (He *et al.* 2009; Qu *et al.* 2012). Gayal has been under studied possibly because it is not domestic cattle and because of its remote distributions (Mohan *et al.* 2007). Most of the plant species it feeds on are not suitable for stall feeding for domesticated cattle (Zhao *et al.* 2003; Deng *et al.* 2007; Xi *et al.* 2011; Mei *et al.* 2016). It feeds on leaves, grasses, bamboo, reeds and other locally available plant species (Mei *et al.* 2016; Memon *et al.* 2018). In contrast to its counterparts, gayal's big body size and meat quality makes it suitable for hybrid breeding to improve meat and milk traits (Ge *et al.* 1996; Giasuddin *et al.* 2003).

Prion diseases are caused by mutation of host-encoded normal cellular prion protein (PrP^{C}) into pathogenic misfolded scrapie prion protein (PrP^{Sc}) (Prusiner *et al.* 1998; Collinge and Clarke 2007). The two noncoding polymorphisms, 23-bp indel in the putative promoter region and 12-bp indel in intron 1 of *PRNP*, regulate the variability of classical BSE in bovine (Sander *et al.* 2004). For instance, the 12-bp indel has been detected to influence resistance to classical BSE in UK Holsteins (Juling *et al.* 2006), while 23-bp indel effects mRNA, the levels of which are detected to be greater in the medulla oblongata of Japanese Black cattle (Msalya *et al.* 2011). However, there is evidence that the susceptibility of a typical BSE is not linked to either of 12-bp and 23-bp indel polymorphisms (Brunelle *et al.* 2007).

The objectives of this study were to identify the allele, genotype and haplotype frequencies of *PRNP* indel polymorphisms in gayal from the Yunnan province of China and compare them with the reported healthy and BSE-affected cattle for evaluation of potential resistance to BSE.

Materials and methods

Animals and sample collection

In total, 225 gayal samples were collected from the Gongshan, China. Physical appearances were considered during sample collection to minimize error of sampling crossbreds. Further, all the analysed samples were first investigated for the presence of mitochondrial lineage of gayal in the previous results (Gou *et al.* 2010; Xi *et al.* 2012). About 5 mL of blood sample from each animal was carefully collected from the jugular vein and were stored at −20°C.

Polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted following a previous protocol (Sambrook *et al.* 1989). The *PRNP* region was amplified using previously described primers by Nakamitsu *et al.* (2006) – forward primer: 5′-CTTCTC TCTCGCAGAAGCAG-3′ and reverse primer: 5′-CCCTT GTTCTTCTGAGCTCC-3′ for the 12-bp indel and by Brunelle *et al.* (2008) – forward primer: 5′-AAGGCACTT CAATCAGTACAC-3′ and reverse primer: 5′-AAGAG TTGGACAGGCACAATG-3′ for the 23-bp indel, respectively. The PCR was carried out in a reaction volume of 25 μL, containing 2.0 μL DNA (~50 ng), 2.0 μL 250 μmol/L dNTPs, 2.5 μL buffer ($MgCl_2$ in the buffer provided by the manufacture), 2.0 μL 10 μmol/L forward primer, 2.0 μL 10 μmol/L reverse primer and 0.2μL10× *Taq* DNA polymerase (5 U/μL, Beijing TransGen Biotechnology, China). PCR cycling conditions were as follows: 5 min at 94°C, 35 cycles of amplification (45 s at 94°C, 45 s at 54°C, 60 s at 72°C) and finally 6 min at 72°C. All PCR products were electrophoresed on 2% agarose gel containing ethidium bromide for visualization. Electrophoresis of the PCR product conducted in either a 191 bp (+) or 168 bp (−) for 23-bp indel polymorphism and in either a 215 bp (+) or a 203 bp (−) for 12-bp indel polymorphism. PCR products were selected randomly and bidirectional sequencing was performed on an ABI 3730x Genetic Analyzer (Applied Biosystems, Foster City, USA).

Data analyses

Genotype and allele frequencies of the gayal *PRNP* promoter and intron 1 variants were analysed manually and deviation from the Hardy–Weinberg (H–W) equilibrium was calculated using the chi-squared test (χ^2) (Oztabak *et al.* 2009; Xi *et al.* 2011). The haplotype frequencies were analysed from the genotype data using Haploview 4.0 software (Barrett *et al.* 2005) and diversity indices were calculated following Nei and Li (1979).

Table 1. Frequency polymorphism of alleles, genotype and haplotype of 23-bp and 12-bp indels of the *PRNP* promoter and intron 1 region in gayal.

	Allele frequency		Genotype frequency				Haplotype frequency			
Locus	+	−	++	+−	−	χ^2	23+/12+	23+/12−	23−/12+	23−/12−
23 bp	0.909	0.091	0.844	0.129	0.027	9.814*	0.789	0.119	0.082	0.01
12 bp	0.667	0.333	0.347	0.64	0.013	43.56**				

χ^2 values were significant in the H–W test.
Level of significance: $^{*}P < 0.01$; $^{**}P < 0.001$.

Table 2. Distribution and comparison of allele and genotype frequencies of 23-bp and 12-bp indels of gayal with BSE-affected and healthy cattle.

23 bp			Allele frequency		*P* value		Genotypic frequency			*P* value	
Breed	Health status	*n*	D23	I23	BSE	Healthy	D23/D23	D23/I23	I23/I23	BSE	Healthy
Gayal		225	0.091	0.909			0.027	0.129	0.844		
German cattle[1]	BSE	43	0.73	0.27	0.000425	–	0.51	0.44	0.05	<0.00001	–
	Healthy	48	0.57	0.43	–	<0.00001	0.35	0.44	0.21	–	<0.00001
German and Swiss[2]	BSE	670	0.68	0.32	<0.00001	–	0.45	0.47	0.09	<0.00001	–
	Healthy	574	0.61	0.39	–	<0.00001	0.37	0.47	0.16	–	<0.00001
12 bp			Allele frequency		*P* value		Genotypic frequency			*P* value	
Breed	Health status	*n*	D12	I12	BSE	Healthy	D12/D12	D12/I12	I12/I12	BSE	Healthy
Gayal		225	0.333	0.667			0.013	0.64	0.347		
German[1]	BSE	43	0.67	0.33	0.000026	–	0.44	0.47	0.09	<0.00001	–
	Healthy	48	0.51	0.49	–	0.0293	0.23	0.56	0.21	–	<0.00001
German and Swiss[2]	BSE	670	0.58	0.42	<0.00001	–	0.34	0.49	0.17	<0.00001	–
	Healthy	574	0.54	0.46	–	0.00001	0.31	0.46	0.23	–	<0.00001

[1]Sander *et al.* (2004); [2]Haase *et al.* (2007). All values found to be significant on comparison of gayal with German BSE-affected, German healthy, German and Swiss BSE-affected and German and Swiss healthy cattle.

Table 3. Comparison of allele frequencies of 12-bp and 23-bp indels among different breeds cattle, buffalo, yak and Gayal.

Species	*n*	Allele frequencies of 12-bp and 23-bp indels				References
		I12	D12	I23	D23	
Cattle (*Bos taurus*)						
German Cattle	48	0.49	0.51	0.43	0.57	Sander *et al.* (2004)
Korean Holstein	52	0.39	0.61	0.30	0.70	Jeong *et al.* (2006)
Korean Hanwoo	107	0.44	0.56	0.40	0.60	Jeong *et al.* (2006)
German Holstein	127	0.57	0.43	0.38	0.62	Juling *et al.* (2006)
German Brown	43	0.86	0.14	0.65	0.35	Juling *et al.* (2006)
Fleckvieh	106	0.38	0.62	0.32	0.68	Juling *et al.* (2006)
Japanese Holstein	278	0.26	0.74	0.21	0.79	Nakamitsu *et al.* (2006)
Japanese Brown	186	0.43	0.57	0.41	0.59	Nakamitsu *et al.* (2006)
USA beef cattle	96	0.37	0.63	0.26	0.74	Clawson *et al.* (2006)
USA dairy cattle	96	0.63	0.47	0.38	0.62	Clawson *et al.* (2006)
Polish Holstein	234	0.46	0.54	0.37	0.63	Czarnik *et al.* (2007)
German Holstein	17	0.47	0.53	0.42	0.58	Kashkevich *et al.* (2007)
Braunvieh	17	0.84	0.16	0.60	0.40	Kashkevich *et al.* (2007)
Fleckvieh	44	0.36	0.64	0.29	0.71	Kashkevich *et al.* (2007)
Abderdeen Angus	99	0.44	0.56	0.27	0.73	Kerber *et al.* (2008)
Charolais	82	0.42	0.58	0.32	0.68	Kerber *et al.* (2008)
Franqueiro	73	0.67	0.33	0.36	0.64	Kerber *et al.* (2008)
Vietnamese Cattle	206	0.52	0.43	0.15	0.85	Muramutsu *et al.* (2008)
South Anatolian Red	50	0.69	0.31	0.36	0.64	Un *et al.* (2008)
East Anatolian Red	50	0.72	0.28	0.40	0.60	Un *et al.* (2008)
Anatolian Grey	50	0.80	0.20	0.62	0.38	Un *et al.* (2008)
Korean Cattle	437	0.44	0.56	0.44	0.56	Kim *et al.* (2009)
Vietnam Native	100	0.93	0.07	0.34	0.66	Shimogiri *et al.* (2010)
Laos Native	72	0.92	0.47	0.37	0.63	Shimogiri *et al.* (2010)
Myanmar Native	110	0.81	0.29	0.20	0.80	Shimogiri *et al.* (2010)
Mongolia Native	44	0.49	0.51	0.30	0.70	Shimogiri *et al.* (2010)
Bangladesh Native	30	0.75	0.25	0.17	0.83	Shimogiri *et al.* (2010)
Chinese Native	349	0.842	0.158	0.404	0.596	Zhao *et al.* (2010)
Hereford	33	0.11	0.88	0.017	0.98	Zhu *et al.* (2011)
Simmental	30	0.19	0.79	0.160	0.830	Zhu *et al.* (2011)
Black Angus	30	0.37	0.63	0.210	0.780	Zhu *et al.* (2011)
Mongolian	31	0.58	0.42	0.480	0.510	Zhu *et al.* (2011)
Polish Holstein	837	0.473	0.527	0.378	0.622	Czarnik *et al.* (2011)
Polish Holstein	651	0.475	0.525	0.381	0.619	Gurgul *et al.* (2012)
Caracu cattle	40	0.700	0.300	0.725	0.275	Galvao *et al.* (2012)
Vietnamese cattle	99	0.95	0.05	0.33	0.67	Uchida *et al.* (2014)
Indonesian cattle	121	0.79	0.21	0.49	0.51	Uchida *et al.* (2014)
Thai cattle	68	0.88	0.13	0.53	0.47	Uchida *et al.* (2014)
Buffalo (*Bubalis bubalis*)						
Anatolian Water Buffalo	106	0.86	0.14	0.92	0.08	Oztabak *et al.* (2009)
Dehong	88	0.99	0.01	0.91	0.08	Zhao *et al.* (2015)
Guangnan	59	1.00	0	0.94	0.06	Zhao *et al.* (2015)
Guangxi	24	0.104	0.896	0.104	0.896	Zhao *et al.* (2015)
Guangdong	73	0.952	0.048	0.027	0.973	Zhao *et al.* (2015)
Chongqing and Guizhou	43	1	0	1	0	Zhao *et al.* (2015)
Fuan and Hainan	25	0.940	0.060	1	0	Zhao *et al.* (2015)
Yak (*Bos grunniens*)						
Chinese Yak	252	0.933	0.067	0.113	0.887	Zhao *et al.* (2009)
Gayal (*Bos frontalis*)						
Mythun (Myanmar)	11	0.77	0.33	0.82	0.18	Shimogiri *et al.* (2010)
Gayal (China)	225	0.67	0.33	0.91	0.09	Present study

The data from previous studies was compared with gayal and it was observed that insertion of alleles was more than deletion thus indicating less susceptibility of gayal to BSE.

Table 4. Comparison of haplotype frequencies for the 23-bp and 12-bp indels between gayal with German BSE-affected and healthy cattle.

Breed	*n*	Haplotype frequency				*P* value	
		I23/I12	I23/D12	D23/I12	D23/D12	BSE	Healthy
German and Swiss BSE- affected	670	0.58		0.1	0.32	<0.00001	–
German and Swiss healthy	574	0.39		0.07	0.54	–	<0.00001
gayal	225	0.789	0.119	0.082	0.01		

All values found to be significant on comparison of gayal with German and Swiss BSE-affected and German and Swiss healthy cattle (Haase *et al*. 2007).

Results

Allele genotype frequencies

We discovered that the allele frequencies of both 23-bp insertion in the promoter region and 12-bp insertion in intron 1 of *PRNP* were significantly high (0.91–23 bp insertion and 0.67–12 bp insertion). This results in significant chi-square values for genotype frequencies (++23 bp and +−12 bp indels) when tested for deviation from the H–W equilibrium (χ^2: 9.81; $P < 0.01$ and χ^2: 43.56; $P < 0.001$) (table 1). Further, the allele and genotype frequencies from our data were compared with both healthy and affected German and Swiss cattle (table 2). The allele and genotype frequencies of 23-bp and 12-bp indel polymorphisms were significantly higher ($P < 0.001$) from healthy as well as affected cattle of both breeds except that allele frequency of 12 bp of German healthy cattle was significantly different ($P < 0.05$) less than ($P < 0.001$) in table 2. Further, the allele frequency of gayal was compared extensively with previous data of cattle, buffalo and yak species (table 3) and we detected that the insertion was higher than the deletion.

Haplotype frequencies

We detected four different haplotypes (23+/12+, 23+/12−, 23−/12+ and 23−/12−) in the *PRNP* promoter and intron 1 of gayal species (table 1). The most frequent haplotype was 23+/12+ with a frequency of 0.79, which was higher than 23−/12+ (frequency=0.08), 23+/12− (frequency=0.12) and 23−/12− (frequency=0.01). The gayal haplotypes were further compared with healthy and affected German and Swiss cattle. The haplotypes of 23-bp and 12-bp indel polymorphisms were also significantly higher ($P < 0.001$) compared with healthy as well as affected cattle of both breeds (table 4).

Discussion

Our study revealed that the allele and genotype frequencies of 23-bp and 12-bp indels were significantly different ($P < 0.05$) from healthy as well as affected cattle of both breeds reported previously (Sander *et al*. 2004; Haase *et al*. 2007) in table 2. This finding is comparable with the previous study on Dehong cattle (Zhao *et al*. 2010). The *PRNP* indel polymorphism allele frequencies of the gayal data were further compared with different species of Bovidae family cattle, buffalo and yak (table 3). Invariant frequency of 12 bp polymorphism vary between 0.11 (Herefod cattle breed; Zhu *et al*. 2011) and 0.99/1.0 (Guangnan, Dehong, Chongqing and Guizhou buffalo breed; Zhao *et al*. 2015). The 12-bp indel polymorphisms in gayal and cattle populations were detected to be similar. In accordance with the previous reports, gayal showed variant of 23 bp polymorphism (frequency: 0.91) close to Anatolian water buffalo. From earlier studies, it is evident that insertion of 23 bp in the putative promoter and a 12 bp in intron 1 is strongly associated with BSE in cattle (Haase *et al*. 2007), buffalo (Oztabak *et al*. 2009) and yak (Zhao *et al*. 2009). However, a contradictory observation on the association of 23 bp in the putative promoter and 12 bp in intron 1 in Hereford cattle was presented by Zhu *et al*. (2011). Our results corroborated with the findings of the previous studies that were conducted on several cattle and buffaloes (Juling *et al*. 2006; Oztabak *et al*. 2009; Zhao *et al*. 2009, 2015) (table 3).

We further characterized and compared the frequency distributions of the 23-bp and 12-bp indel polymorphisms of gayal with healthy and BSE cattle. This includes extensively reported data of healthy cattle and BSE cattle (Sander *et al*. 2004; Seabury *et al*. 2004; Jeong *et al*. 2006; Juling *et al*. 2006; Nakamitsu *et al*. 2006; Brunelle *et al*. 2007, 2008; Czarnik *et al*. 2007, 2011; Haase *et al*. 2007; Kerber *et al*. 2008; Muramutsu *et al*. 2008; Un *et al*. 2008; Kim *et al*. 2009; Msalya *et al*. 2009; Murdoch *et al*. 2010; Shimogiri *et al*. 2010; Zhao *et al*. 2010; Qin *et al*. 2011; Zhu *et al*. 2011; Galvao *et al*. 2012; Gurgul *et al*. 2012; Uchida *et al*. 2014). The results showed that the 23 bp and 12 bp allele frequencies in BSE cattle were significantly higher ($P < 0.001$) than gayal (figure 1, a&c). Moreover, the genotype frequencies of 23 and 12 bp were significantly different from healthy as well as affected cattle (figure 1, b&d). Further, the haplotype frequencies were also significant for both healthy and affected cattle (figure 1e). It was reported earlier that susceptibility to BSE was strongly associated with 12 bp deletion or D23–D12 haplotype

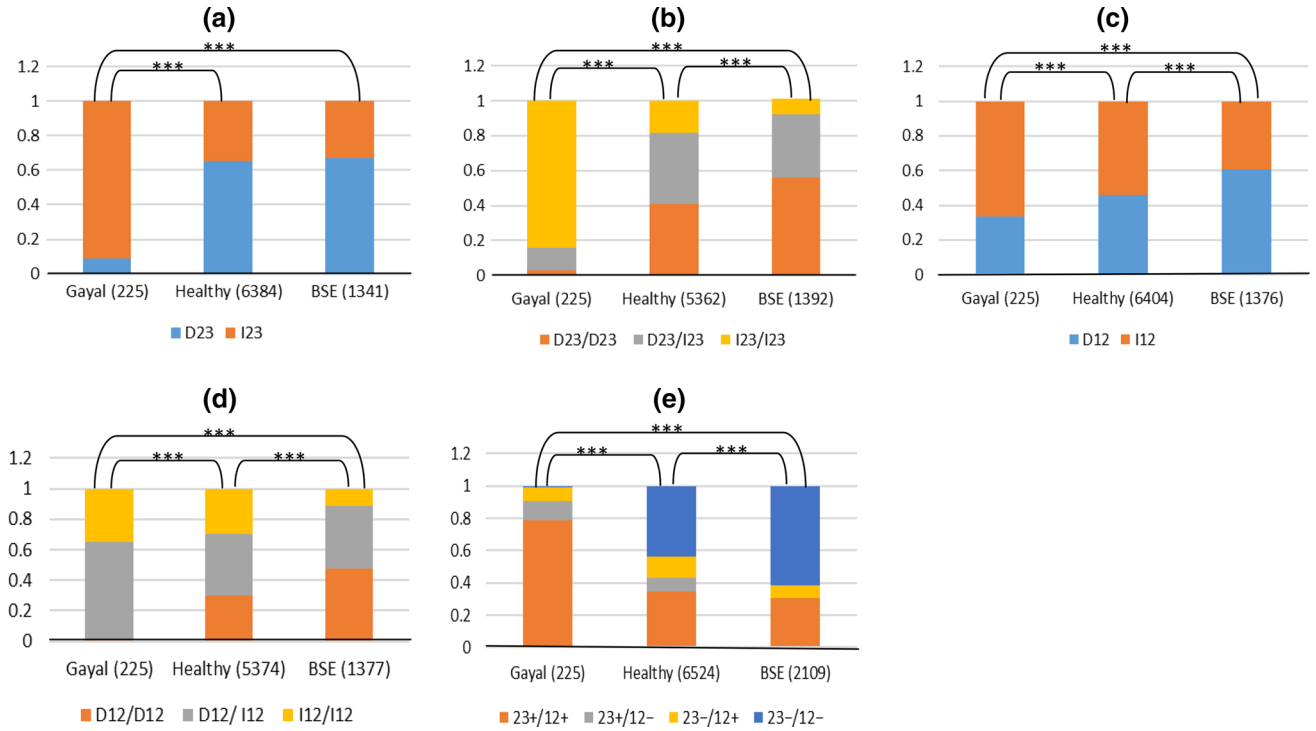

Figure 1. Comparison of the frequency distributions of gayal with different healthy and BSE cattle. (a) and (b) Show the allele and genotype distributions in the 23-bp indel polymorphism, while (c) and (d) indicate the allele and genotype frequencies in the 12-bp indel polymorphism, respectively. (e) Corresponds to the haplotype distributions assembled by the two indels. ***Highly significant differences ($P < 0.001$) in frequency distribution.

(Juling *et al.* 2006). The low haplotype distribution of D23–D12 haplotypes indicated less susceptibility of Chinese cattle to BSE (Zhao *et al.* 2010). This is similar to our results with a lower haplotype D12–D23 frequency (0.01) showing lower tendency toward susceptibility.

Further, we compared our results with those reported for other species which have not been found with and probably never exposed to BSE. Our results showed that haplotype D23–D12 was lower than buffalo and yak (0.027 versus 0.05 and 0.813, respectively) (Oztabak *et al.* 2009; Zhao *et al.* 2009). More interestingly, the 23 bp or 12 bp insertion frequencies in our gayals of the Yunnan province of China were found even more favourable for protection against BSE infection than in yak and buffalo. However, only intracerebral and oral challenge studies with BSE in cattle or gayals could serve as real proof that the *PRNP* 23 bp/12 bp insertions are valid for application in breeding practice.

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